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13. ABSTRACT *(Maximum 200 Words)*

We have been developing conditionally replicative adenoviral agents (CRADs) for prostate cancer gene therapy. We have sought to improve these agents by increasing their infectivity for tumor targets and by enhancing the specificity of their replication exclusively within tumor targets.

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A. INTRODUCTION

Replicative adenoviral vectors (CRAD) represent a promising therapeutic approach which has been applied recently in context of cancer of the prostate. As for the non-replicative cancer gene therapy approaches, however, the efficacy of the replicative strategies is subservient to vector-mediated tumor transduction. In this regard, one of the attractive features of the CRAD approach is that it capitalizes on the unparallel efficiency of adenoviral vectors (Ad) in accomplishing *in vivo* transduction. Indeed, of all of the currently available vector approaches, Ad vectors possess the highest capacity to achieve *in situ* transduction of tumor. Despite this capacity, overall efficacy of Ad-based cancer gene therapy approaches remain limited by suboptimal vector efficiency. Of note, human trials carried out to date have demonstrated relatively inefficient gene transfer to tumor achieved by Ad vectors employed in *in vivo* delivery schemas. This has been understood to result from a relative paucity of the primary adenovirus receptor, coxsackie-adenovirus receptor (CAR), on tumor cells relative to their cell line counterparts. Indeed, a relative paucity of CAR has been shown to limit Ad vector efficacy in a number of tumor contexts, possibly representing a fundamental practical barrier to realizing the full benefit of this vector system for cancer gene therapy applications. On this basis, it has been proposed that gene delivery via "CAR-independent" pathways may be required to circumvent this key aspect of tumor biology. Especially noteworthy in this regard, has been the observation in the Onyx trials of transductional-barriers limiting overall efficacy of CRAD-based replicative approaches. Thus, it is clear that augmenting the gene transfer efficacy of Ad vectors is essential to deriving their full benefit in the context of the conceptually promising CRAD strategies. To this end, we have developed novel approaches to alter the tropism of Ad vectors such that CAR-independent gene transfer may be achieved. Such CAR-independent gene transfer allows dramatic augmentations of the gene transfer efficacy of the Ad vector, especially in context whereby CAR levels are limiting. We thus propose to develop such modifications in the context of CRAD to improve its infection efficacy for tumor cells. We hypothesize that such modifications will overcome biological limits of tumor noted in human clinical trials and thereby allow full realization of the potential benefits of the CRAD approach. The demonstration of this principle, in model systems, would allow a rapid translation of this strategy into the human clinical context for prostate cancer therapy.

B. BODY

We are endeavoring in this proposal to improve conditionally replicative adenoviruses (CRADs) as anti-tumor agents. In this regard, CRADs function as anti-tumor agents via specific replication within tumor cells whereby direct target cell oncolysis is achieved. Key to realizing this end is effective viral infection of tumor cells – initially in the context of the primary tumor inoculum of the CRAD, and secondarily in the context of lateralization of the new virions produced via successive rounds of replication. Of note in this regard, it has recently been recognized that tumor cells are frequently resistant to adenovirus infection based on a relative paucity of the primary adenoviral receptor, coxsakie-adenovirus-receptor ("CAR"). This key aspect of tumor biology would thus mitigate against CRAD function, based on inefficient target cell infection.

We have sought to address this issue by developing adenoviral agents which can infect tumor cells via CAR-independent mechanisms. Two strategies have been endeavored to this end. First, we have accomplished genetic capsid modifications based on incorporation of heterologous targeting ligands. Specifically, we have configured the integrin binding peptide RGD4C into the HI loop of the adenovirus fiber knob protein. This was accomplished in the context of the CRAD agent "delta 24", which replicates selectively in target cells with a defective retinoblastoma (Rb) axis. Our studies showed that the HI loop infectivity enhancement maneuver allowed improved target cell oncolysis via the delta 24 CRAD. Of note, direct improvements in tumor cell killing could be shown in in vitro, and in vivo model systems. Further, therapeutic results were achieved via distinct delivery schemas – intratumoral and systemic vascular. As well, we have created knob chimeras whereby the type 5-adenovirus base of the CRAD agent is modified to contain the knob of an alternate, non-type 5 serotype. Such chimeras also accomplished CAR-independent gene transfer with improved oncolysis via the corresponding CRAD. Thus, two distinct methods of achieving CAR-independent infection via CRAD agents have been accomplished. These studies have established the principle that such infectivity enhancement maneuvers can directly improve the therapeutic potency of CRAD agents (Appendix A & B).

We have also sought to improve the therapeutic index of CRAD agents via improving anti-tumor specificity. To this end, we have employed tumor selective promoters (tsps) to control expression of key adenoviral early genes. For the current application, an idea tsp would exhibit a "tumor on/liver off" phenotype as a means to compensate for the natural hepatotropism of systemically administered adenovirus. This function of candidate promoters can only be validly tested in in vivo model systems whereby systemic vector administration in endeavored. We have thus derived adenovirus vectors (Ad) whereby reporter gene expression is controlled via candidate promoters for evaluation in a systemic delivery context. To this end, we evaluated two novel promoter elements – the regulatory control regions of the cyclooxygenase-2 (cox-2) and midkine (Mk) genes (Appendix C & D). Analysis in human tumor cell lines validated that the cox-2 and Mk promoters were capable of driving levels of transgene expression comparable to the powerful viral CMV promoter. Importantly, systemic administration of Ad vectors validated that these candidate promoters exhibited the key "liver off" phenotype. These findings thus establish that these promoter elements may be of utility for deriving prostate cancer CRAD agents with an improved selectivity profile. Such improvements in selectivity may serve as an important means to improve the therapeutic index of these agents. CRADs employing the cox-2 and midkine promoters are currently being derived. These will be studied in stringent model systems for therapeutic utility.

We have thus addressed, in this first funding period, two key aspects of CRAD physiology – replicative specificity and infection efficiency. Our studies have allowed the derivation of a new class of CRAD agents which embody augmented potency based on infectivity enhancement. In addition, our studies have identified key promoter elements compatible with systemic employment of CRAD

agents. Further studies will employ these methods, in combination, to determine the direct efficacy gains accrued.

C. KEY RESEARCH ACCOMPLISHMENTS

- We have established that CRAD agents can be genetically modified to incorporate heterologous peptides in the HI loop of the fiber knob

- We have established that such modifications allow CAR-independent infection of target cells

- We have established that such CAR-independent infection allows enhanced oncolytic potency

- We have applied two novel promoter elements (midkine and cyclooxygenase-2) for prostate cancer

- We have shown that these promoters have high levels of inducibility in prostate cancer cell lines

- We have shown that these promoters are non-inducibile in non-prostate normal tissues

- These findings have established the basis of construction of an ideal CRAD agent for prostate cancer based on the incorporation of the aforementioned infectivity-enhancement and transcriptional control (midkine, cox-2) elements

D. REPORTABLE OUTCOMES

Manuscripts

1. Balague C, Noya F, Alemany R, Chow LT, and **Curiel DT**. Human papillomavirus E6E7-mediated adenovirus oncolysis: selectivity of mutant adenovirus replication ascertained in organotypic cultures of human keratinocytes. (Submitted, 2000)
2. Wesseling JG, Yamamoto M, Adachi Y, Bosma PJ, van Wijland M, Blackwell JL, Li H, Reynolds PN, Dmitriev I, Vickers SM, Huibregtse K, and **Curiel DT**. Midkine and cyclooxygenase-2 promoters are promising for adenoviral vector gene delivery of pancreatic cancinoma. (Submitted, 2000).
3. de Gruijl TD, Luykx-de Bakker SA, Tillman BW, van den Eertwegh AJM, van der Bij G, Safer M, Haisma HJ, **Curiel DT**, Scheper RJ, Pinedo HM, and Gerritsen WR. CD40-targeted adenoviral gene transfer to human cutaneous dendritic cells *in situ*. (Submitted, 2000)
4. Casado Saenz E, Nettelbeck DM, Gomez-Navarro J, Gonzalez Baron M, Siegal GP, Barnes MN, Alvarez RD, and **Curiel DT**. Transcriptional targeting for ovarian cancer gene therapy. (Submitted, 2000).

5. **Curiel DT**. The development of conditionally replicative adenoviruses for cancer therapy. *Clinical Cancer Research* 6:3395-3399, 2000.
6. Nicklin SA, Reynolds PN, Brosnan MJ, White SJ, **Curiel DT**, Dominiczak AF, and Baker AH. Analysis of cell-specific promoters for viral gene therapy targeted at the vascular endothelium. (In Press, *Journal of Hypertension*)
7. Alemany R, Balague C, and **Curiel DT**. Replicative adenoviruses for cancer therapy. *Nature Biotechnology* 18:723-727, 2000.
8. Suzuki K, Fueyo J, Krasnykh V, Reynolds PN, **Curiel DT**, and Alemany R. A conditionally replicative adenovirus with enhanced infectivity shows improved oncolytic potency. *Clinical Cancer Research* 7:120-126, 2001.
9. Gomez-Navarro J, and **Curiel DT**. Targeted Vectors for cancer gene therapy. (Submitted, 2000).
10. Dmitriev I, Kashentseva E, Rogers BE, Kransykh V, and **Curiel DT**. Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor-positive cells. *Journal of Virology* (74)15:6875-6884, 2000.
11. Reynolds PN and **Curiel DT**. Strategies to adapt adenoviral vectors for gene therapy applications: targeting and integration. *The Development of Human Gene Therapy*, Cold Spring Harbor Laboratory Press:111-130, 1999.

Presentations

Gene Therapy for Prostate Cancer, CapCure Foundation, Atlanta, Georgia, April 18, 2000.

American Society of Gene Therapy 3rd Annual Meeting, "Meet the Professor Breakfast", Denver, Colorado, June 3, 2000.

American Society of Gene Therapy 3rd Annual Meeting, Scientific Symposium, Viral Vectors: Novel Developments, (Invited Speaker), Denver, Colorado, June 4, 2000.

American Association for Cancer Research 92nd Annual Meeting, Experimental Gene Therapy, New Orleans, LA, March 24-28th, 2001.

Patents

Infectivity-Enhanced Conditionally Replicative Adenovirus and Uses Thereof; Curiel, D6219

Adenoviral Vector Containing Cyclooxygenase-2 Promoter And Uses Thereof; Curiel, D6384

E. CONCLUSIONS

Our findings have established key facets of CRAD biology which have direct implications on their design/utility. Specifically, infectivity-enhancement maneuvers substantially improve the potency of these agents and enhance their anti-tumor capacity. In addition, we have defined novel regulatory elements relevant to prostate cancer CRAD design. These elements exhibit an ideal inductivity/specificity profile for the current application. In the aggregate, these advancements will allow the rapid derivation of a CRAD agent for prostate cancer with optimized biologic properties predicating anti-tumor efficacy.

APPENDIX A

A Conditionally Replicative Adenovirus with Enhanced Infectivity Shows Improved Oncolytic Potency[1]

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ABSTRACT

The absence or the presence of low levels of the Coxsackievirus and adenovirus receptor (CAR) on several tumor types might limit the efficacy of recently proposed tumor-specific or conditionally replicative adenoviruses (CRAds). To address this issue, we used a genetic modification of the fiber knob in the context of an E1A-defective CRAd to allow CAR-independent target cell infection as a means to enhance oncolytic potency. Such infectivity-enhanced CRAd showed higher replication, more efficient infection, and lysis of tumor cells *in vitro*. Of note, the improved antitumor effect of the fiber-modified CRAd could be demonstrated *in vivo*. We conclude that the combination of genomic modification to achieve tumor-selective replication and capsid modification to enhance infectivity yields more potent oncolytic adenoviruses for use in cancer treatment.

INTRODUCTION

CRAds[3] represent a novel and promising approach for treating neoplastic diseases (1, 2). The use of CRAds offers two advantages over conventional gene therapy. First, CRAds have an intrinsic amplification capacity that allows extensive tumor infection, leading to expansive oncolysis by reason of the actual cytopathic effect of the virus. Second, the restriction of viral replication to tumors avoids damage to normal host tissues and improves the therapeutic index. Two strategies have been implemented to achieve specificity: the control of the expression of an essential early viral gene by using tumor-specific promoters (3); and deletions in viral genes encoding proteins that interact with cellular proteins necessary to complete the viral lytic life cycle in normal cells, but not in tumor cells (4). Both CRAd-based strategies have been rapidly translated into clinical trials (5, 6).

However, realization of the full utility of CRAds in cancer therapeutics depends on their ability to infect human tumors. Previous studies on adenovirus-mediated gene delivery to human tumor cells have pointed out the highly variable expression of primary adenoviral receptor, CAR, in neoplastic cells (7, 8), and this variation may curtail the initial infection and lateral propagation of CRAds (9). On the basis of these data, it has been proposed that gene delivery via CAR-independent pathways is required to overcome this aspect of tumor biology (10, 11). We have focused on α_V integrins as enhancers of adenoviral infection according to a previous report on the correlation of the levels of α_V integrins expressed by tumor cells with the efficiency of adenovirus-mediated gene transfer (12). Furthermore, previous studies demonstrate that α_V integrins are aberrantly expressed in several types of cancer (13, 14) and are present in tumor blood vessels of breast cancer and malignant melanoma (15).

Modifications of capsid proteins responsible for adenovirus binding to target cells can alter its tissue tropism. These data favor the incorporation of an Arg-Gly-Asp (RGD) sequence, known to interact with α_V integrins, into the adenovirus fiber to enhance tumor infection. Recently, we developed an approach based on the genetic incorporation of a sequence encoding an RGD peptide into the HI loop of the fiber knob. The addition of RGD-integrin interactions on primary CAR binding confers an expanded tropism to the fiber-modified adenovirus, and this effect has been demonstrated in previous studies (16, 17).

In this study, we combined the fiber knob modification strategy with a CRAd based on a partial deletion of the *E1A* gene, which synthesizes a defective protein unable to bind host cell Rb protein. The selectivity of this mutant adenovirus has been previously demonstrated by Fueyo *et al.* (18) and recently by another group that uses a virus with the same deletion (19). Our results demonstrated that the incorporation of the RGD motif into the fiber of a CRAd enhances its oncolytic potency *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Lines

A549 human lung adenocarcinoma and LNCaP human prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines are defective

[3] The abbreviations used are: CRAd, conditionally replicative adenovirus; BrdUrd, bromodeoxyuridine; CAR, Coxsackievirus and adenovirus receptor; i.t., intratumoral/intratumorally; Rb, retinoblastoma; FBS, fetal bovine serum; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide.
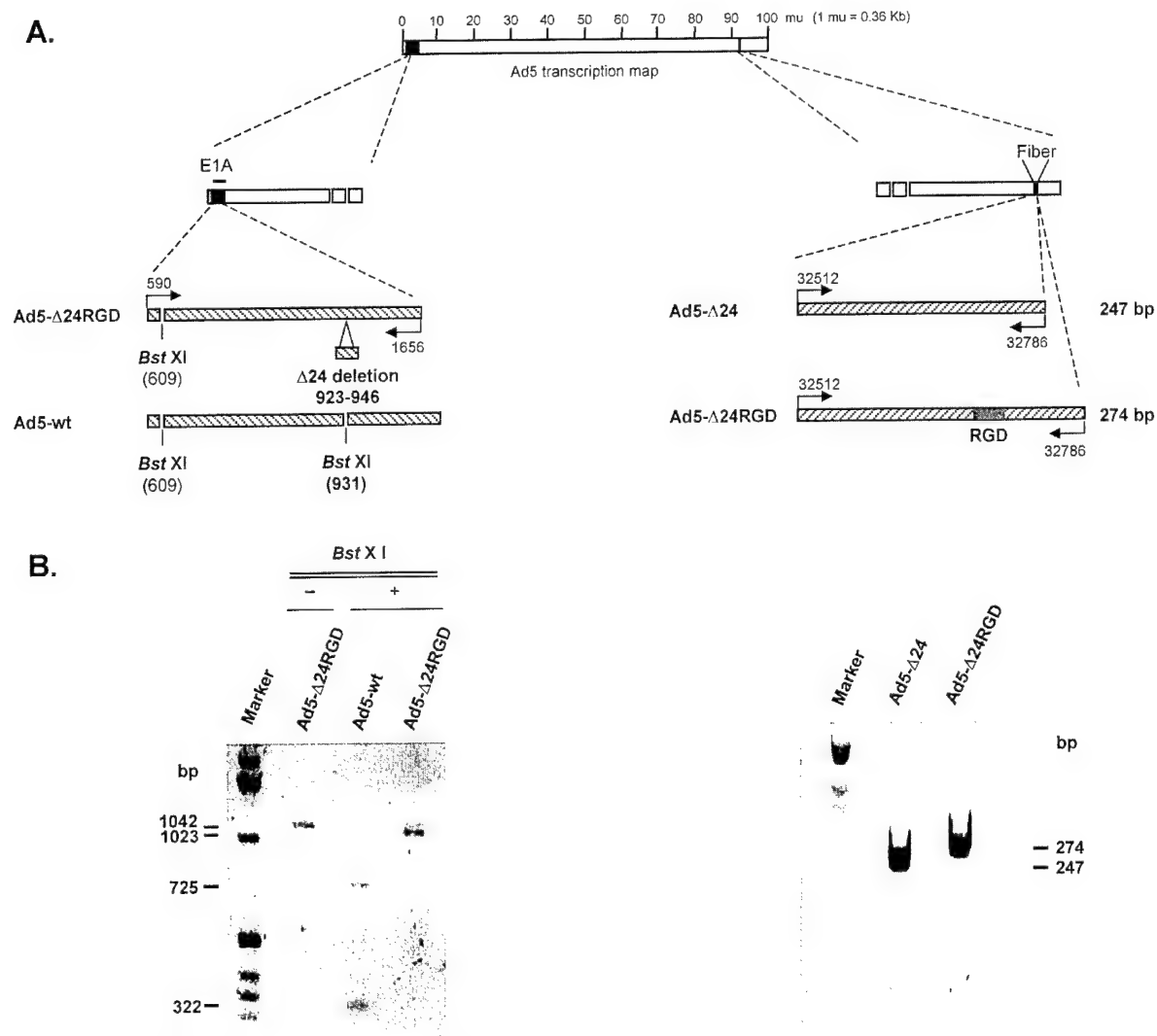
Fig. 1 Analyses of adenoviral DNA. *A*, map of E1A- and fiber-encoding regions of Ad5-Δ24RGD amplified by PCR, showing the 24-bp deletion and the introduced RGD-encoding sequence. *B*, restriction analysis of Ad5-Δ24RGD. The presence of the 24-bp deletion was confirmed by *Bst*XI digestion of the PCR product of the E1A region. The fragments were resolved on a 2% agarose gel, and visualized by UV fluorescence. *Left, Marker* (Life Technologies, Inc.), 1-kb DNA ladder. The presence of uncleaved PCR product verified the presence of the deletion. PCR amplification products of the region encoding the fiber from Ad5-Δ24 and Ad5-Δ24RGD were resolved on a 6% acrylamide gel. *Right, Marker* (Life Technologies, Inc.), 100-bp DNA ladder. The bigger size (27 bp) of Ad5-Δ24RGD band indicates the presence of the sequence encoding RGD.

in the Rb pathway because of a deficiency in $p16^{INK4}$ (20–22). The cells were cultured in DMEM supplemented with 5% heat-inactivated FBS, 100 I.U./ml penicillin, and 100 µg/ml streptomycin.

Virus Construction

Ad5-Δ24 Mutant. The replication-competent Ad5-Δ24 adenovirus was provided by J. F. (The University of Texas M. D. Anderson Cancer Center, Houston, TX). This virus contains a 24-nucleotide deletion, from Ad5 bp 923 to 946 (both included), corresponding to the amino acid sequence $L_{122}TCHEAGF_{129}$ of the E1A protein known to be necessary for Rb protein binding (23). Details of the tumor-specific replication of this virus are presented elsewhere (18, 19).

RGD Modification of Ad5luc and Ad5-Δ24. Ad5lucRGD is an E1-deleted virus containing the recombinant RGD fiber and expressing the firefly luciferase. This vector was constructed by homologous recombination of the E1 region containing the *luciferase* gene into the plasmid pVK503 that contains the modified fiber (15). A similar procedure was followed to construct the RGD-modified version of Ad5-Δ24. Briefly, an E1 fragment containing the 24-bp deletion was isolated from the plasmid pXC1-Δ24, originally used to construct Ad5-Δ24 (18), and cloned by homologous recombination into the *Cla*I–digested plasmid pVK503 containing the RGD fiber (15). The genome of the new virus was released from the plasmid backbone by digestion with *Pac*I, and the resulting fragment was used to transfect 293 cells to rescue the Ad5-

Δ24RGD. The presence of the RGD motif in Ad5-Δ24RGD and Ad5lucRGD was confirmed by PCR with the fiber primers FiberUp (5'-CAAACGCTGTTGGATTTATG-3') and Fiber-Down (5'-GTGTAAGAGGATGTGGCAAAT-3'). The Δ24 deletion was analyzed by PCR with primers E1a-1 (5'-ATTAC-CGAAGAAATGGCCGC-3') and E1a-2 (5'-CCATTTAA-CACGCCATGCA-3') followed by *Bst*XI digestion.

Virus DNA Replication. A549 cells, cultured in 6-well plates, were infected with Ad5-Δ24 or Ad5-Δ24RGD at a dose of 0.01 viral particles/cell. The cells were maintained in DMEM-5% FBS with 1 μCi/ml BrdUrd (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Attached and detached cells were harvested at 2, 4, 6, and 8 days after infection, and encapsidated viral DNA was purified by the spermine-HCl method (24). The DNA was digested with *Hin*dIII and resolved in 1% agarose gel. The BrdUrd incorporated into the DNA resulting from viral replication was detected by Southwestern blot using mouse anti-BrdUrd IgG (DAKO, Carpinteria, CA) and peroxidase-labeled antimouse antibody (Amersham). The membrane was exposed to Kodak Biomax ML film and developed in an automated processor.

Adenovirus Yield Assay. A549 cells cultured in 6-well plates were infected with 0.01 particle/cell Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD, and maintained in DMEM-5% FBS. After 8 days, cells and media were harvested, and the titer was determined by plaque assay.

Oncolysis Assay. A549 and LNCaP cells cultured by triplicate in 6-well plates were infected with one of the three types of adenovirus at doses of 0.001 or 0.01 viral particles/cell. Eight (A549) and 10 (LNCaP) days after infection, the cells were fixed and stained with crystal violet solution.

***In Vitro* Cytotoxicity Assay (XTT).** A549 and LNCaP cells were seeded and infected in parallel with the ones used for the oncolysis assay described above. Eight and 10 days after infection, cell survival was determined using XTT (Sigma, St. Louis, MO). The number of living cells was calculated from noninfected cells cultured and treated with XTT in the same way as were the experimental groups.

s.c. Tumor Xenograft Model in Nude Mice. Female athymic nu/nu mice (Frederick Cancer Research, Frederick, MD), 8–10 weeks old, were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Eight million A549 cells were xenografted under the skin of each flank in anesthetized mice. When the nodules reached 60–100 mm^3, a single dose of 10^9 viral particles (high-dose experiment; $n = 5$) or 10^7 viral particles (low-dose experiment; $n = 4$) of Ad5lucRGD, Ad5-Δ24, Ad5-Δ24RGD, or PBS was administered i.t. Tumor size was monitored twice a week, and fractional volume was calculated from the formula: (length × width × depth) × 1/2. The mice were euthanized 35 days after the treatment because of the size of the tumors in the control group. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Statistical differences among groups were assessed with Student's t tests.

Adenovirus Hexon Immunodetection. The presence of adenovirus hexon in the treated tumor xenografts was assessed by immunofluorescence. A549 tumor sections were treated with
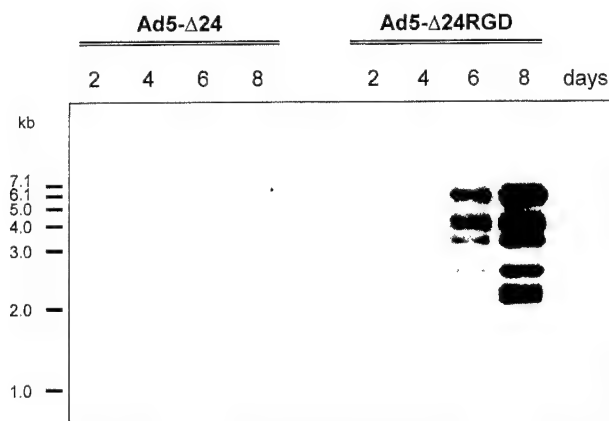

Fig. 2   Propagation efficiency of Ad5-Δ24 *versus* Ad5-Δ24RGD. A549 cells were infected with 0.01 particles/cell Ad5-Δ24 or Ad5-Δ24RGD and incubated in medium containing 1 μCi/ml BrdUrd. At the indicated times after infection, the cells were harvested, and the encapsidated DNA was purified by the spermine-HCl method. Viral DNA from 6 × 10^5 infected cells was digested with *Hin*dIII and electrophoresed, and the resulting fragments were blotted into a membrane that was processed with a mouse anti-BrdUrd antibody. The amount of BrdUrd incorporated into viral DNA indicated that Ad5-Δ24RGD propagation is more efficient than that of Ad5-Δ24.

goat antihexon (Chemicon Inc., Temecula, CA) and Alexa Fluor 488-labeled donkey antigoat (Molecular Probes, Eugene, OR) antibodies, and were counterstained with Hoechst 33342 (Molecular Probes). The slides were analyzed under a fluorescent microscope (Leitz Orthoplan).

RESULTS

Propagation Advantage of an RGD-modified CRAd. The Δ24 deletion of E1A and the RGD insertion in the fiber knob were combined into a unique viral genome by homologous recombination, and the resulting Ad5-Δ24RGD was propagated efficiently in A549 cells. The 24-bp deletion in the *E1A* gene and the RGD-encoding sequence in the fiber were verified by PCR (Fig. 1). Of note, no adenoviruses having wild-type E1 or wild-type fiber appeared throughout the propagation of Ad5-Δ24RGD, a finding that confirms the lack of endogenous adenoviral sequences in A549 cells.

After structural confirmation, the replication capacity of Ad5-Δ24RGD and Ad5Δ24 was compared. A549 cells were infected with 0.01 viral particle per cell of each virus and were maintained in medium with BrdUrd throughout the 8-day incubation period. The encapsidated viral DNA was purified on days 2, 4, 6, and 8 postinfection, and the samples were analyzed by Southwestern blot as described in "Materials and Methods." As indicated by the BrdUrd incorporated into replicating viral DNA, Ad5-Δ24RGD propagation was more efficient than that of Ad5-Δ24 (Fig. 2). The Ad5-Δ24RGD DNA can be detected not only sooner (day 6) compared with Ad5-Δ24 DNA (day 8) but in greater amounts. Thus, the infectivity advantage conferred by RGD incorporation into the fiber knob increased adenovirus propagation in target cells.

Increased Viral Yield of Infectivity-enhanced CRAd *in Vitro*.   On the basis of the previous experiment, we decided to compare the amount of infectious virus produced by
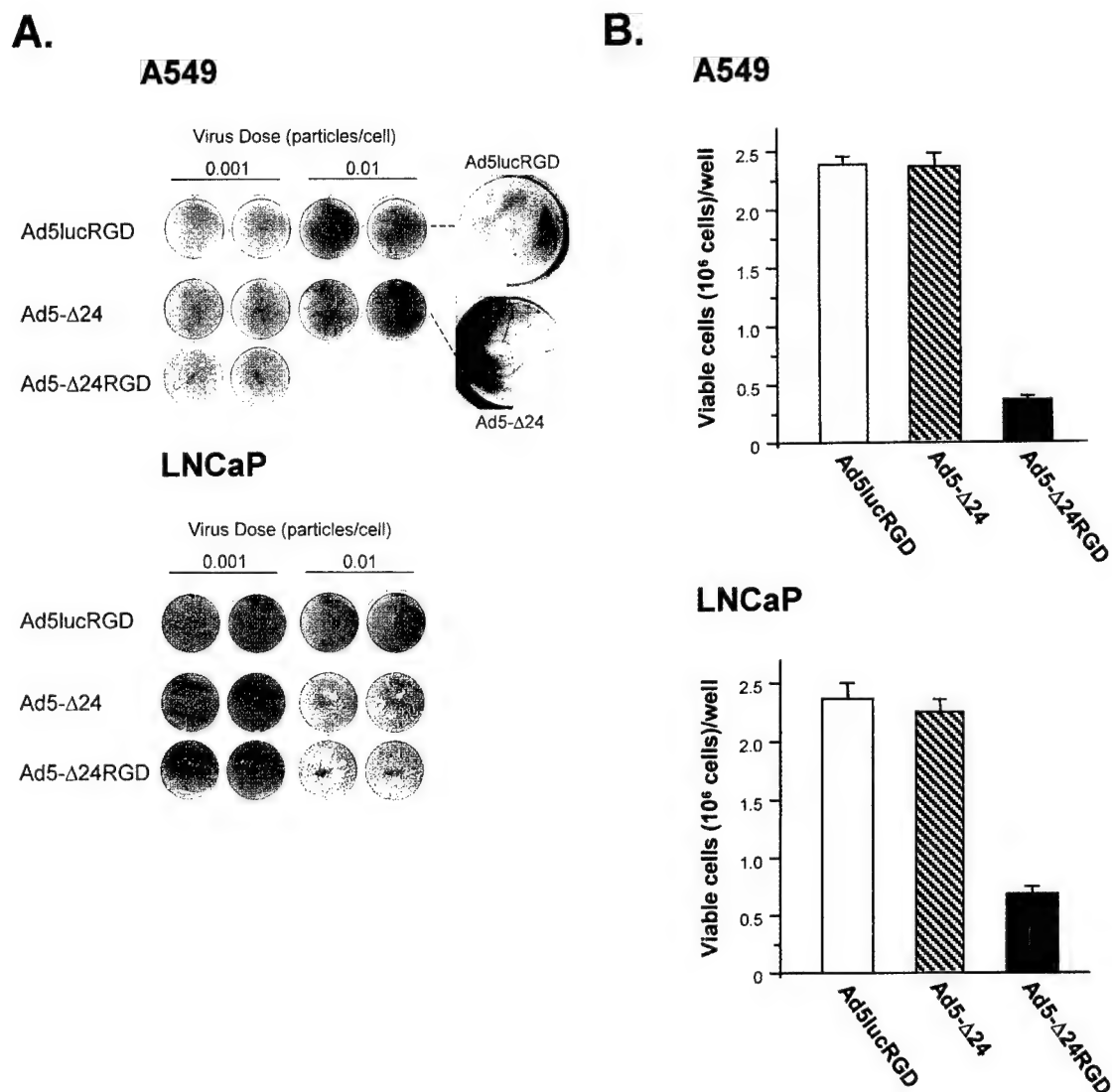
A.

A549

Virus Dose (particles/cell)

LNCaP

Virus Dose (particles/cell)

B.

A549

LNCaP

Fig. 3 Oncolytic potency of the RGD-modified virus. In *A*, A549 and LNCaP cells were infected with 0.001 or 0.01 particles/cell Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD. Eight (*A549*) and 10 days (*LNCaP*) later, the cells were fixed and stained with crystal violet. A higher magnification of two wells is presented to show the incipient cytopathic effect of Ad5-Δ24. In *B*, in parallel, cell viability was analyzed with an XTT colorimetric assay. In both cell lines, Ad5-Δ24RGD had higher lytic potency than did its unmodified counterpart, as shown by the percentage of viable cells remaining in the corresponding treatment conditions.

Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD in A549 cells at 8 days after infection by plaque assay. Ad5-Δ24RGD produced a viral yield of 3.75×10^9 plaque-forming units/ml which was 43 times higher than that of its unmodified Ad5-Δ24 counterpart (8.75×10^7 plaque-forming units/ml). No virus was obtained from the nonreplicative control Ad5lucRGD-infected cells. These results are consistent with the fact that modifying the fiber knob with an RGD motif led to enhancement of viral infectivity and an increase in the production of infectious adenovirus.

Increased Oncolytic Potency of Infectivity-enhanced CRAd *in Vitro*. To demonstrate the increased lytic potency of Ad5-Δ24RGD, we infected A549 and LNCaP cells with small amounts of each virus to allow multiple cycles of viral replica-

tion over the ensuing 8 days, then stained the attached cells with crystal violet and counted viable cells by XTT assay. In both cell lines, the fewest viable cells were detected in the Ad5-Δ24RGD-infected group (Fig. 3, *A* and *B*). The cell lysis capacity of Ad5-Δ24RGD is 7 times higher in A549, and 3.5 times higher in LNCaP compared with Ad5-Δ24. These results demonstrate that the fiber knob modification enhanced adenoviral lytic potency over that of the Ad5-Δ24 virus.

Increased Oncolytic Potency of Infectivity-enhanced CRAd *in Vivo*. The ultimate goal of this study was to demonstrate the oncolytic superiority of infectivity-enhanced CRAds over that of unmodified adenoviruses *in vivo*. Because low doses of virus allow several cycles of replication along with destruction of tumor cells, even a single dose

A.

B.

C.

a   b   c
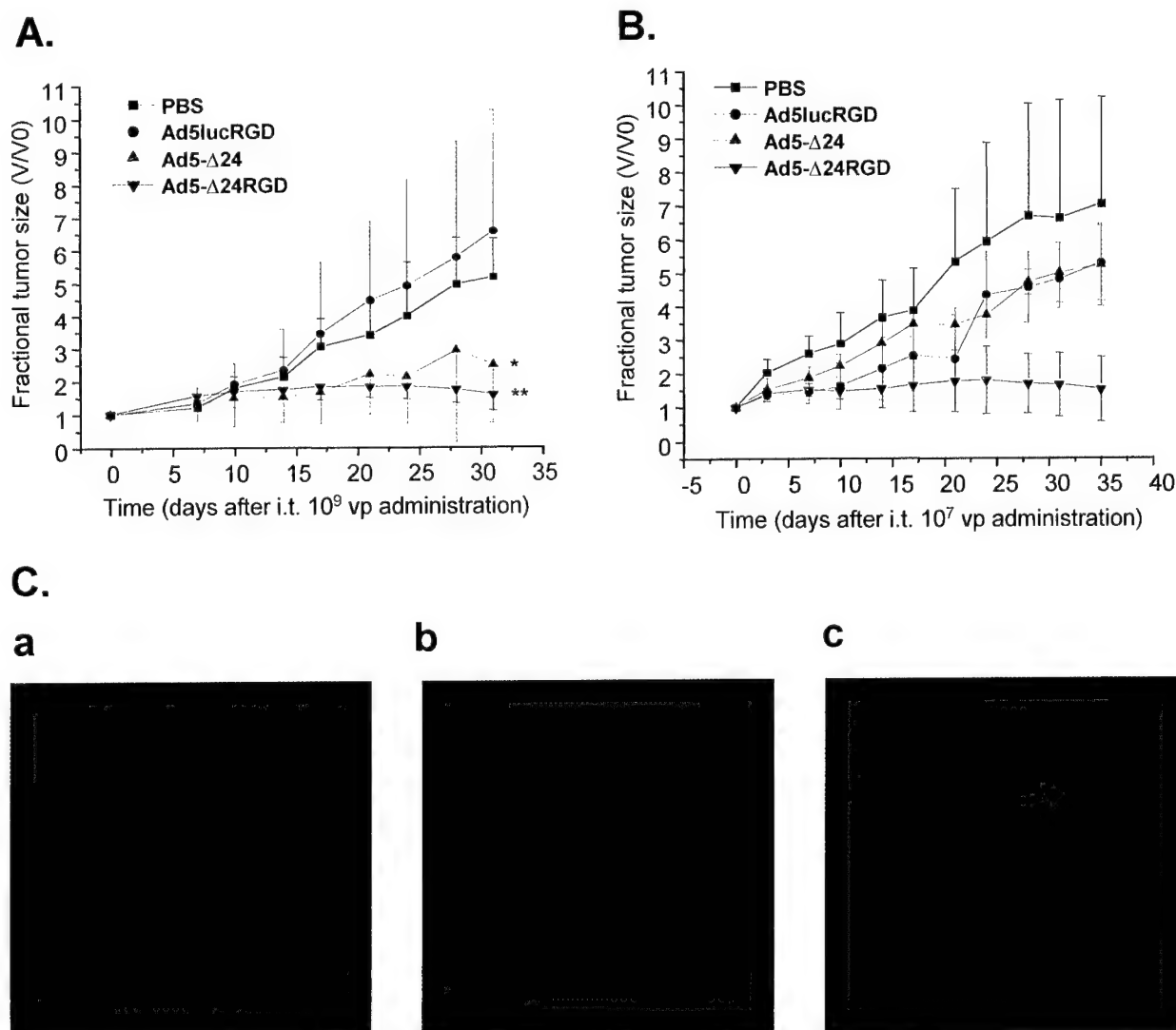
Fig. 4   In vivo oncolysis by high and low doses of infectivity-enhanced CRAds. s.c. A549 xenografts in nude mice were treated with a single i.t. injection of (A) 10^9 viral particles or (B) 10^7 viral particles of Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD, or with PBS alone. Tumor size was measured twice a week. Results are shown as fractional tumor volumes (*V/V0*, where V = volume at each time point, and $V0$ = volume at adenovirus injection); *each line*, the mean of five tumors (±SD) in the high-dose group, and 4 tumors (±SD) in the low-dose group. In the high-dose experiment, both CRAds show a similar oncolytic effect that results in smaller tumors compared with PBS-treated groups (∗, Ad5-Δ24, $P < 0.05$; ∗∗, Ad5-Δ24RGD, $P < 0.01$). However, in the low-dose experiment, tumors treated with Ad5-Δ24 followed a growth curve similar to that of tumors treated with nonreplicative Ad5lucRGD, whereas tumors treated with Ad5-Δ24RGD did not grow ($P < 0.01$ compared with PBS). *C*, detection of adenovirus hexon in tumor xenografts by immunofluorescence. Frozen sections of tumor specimens injected with (*a*) Ad5lucRGD, (*b*) Ad5-Δ24, and (*c*) Ad5-Δ24RGD were treated with goat antihexon antibody and Alexa Fluor 488-labeled donkey antigoat antibody, and nuclei were counterstained with Hoechst 33342. Images were captured from Leitz fluorescence microscope (×100) with a double filter. Sections taken from tumors treated with CRAds were positive for adenovirus presence (*green dots in b* and *c*); Ad5-Δ24RGD signal was stronger than that of Ad5-Δ24. Samples taken from tumors treated with PBS (not shown) or Ad5lucRGD exhibited no hexon signal (*a*). *vp*, viral particles; *Ad*, adenovirus.

would produce an exponential rise in the number of killed cells, which would extend to the entire tumor. To demonstrate this hypothesis, we treated A549 xenografts in nude mice with a single i.t. injection (10^9 viral particles) of one of the three viruses or with PBS. At 32 days after injection, both CRAds had an oncolytic effect in the tumors opposite to that of those treated with nonreplicative virus or with PBS (Ad5-Δ24, $P < 0.05$; Ad5-Δ24RGD, $P < 0.01$ compared with PBS group; Fig. 4A). Given these results, we conducted another

experiment in which we administered a 100-fold lower dose (10^7 viral particles) of the viruses. At this dose, Ad5-Δ24 treatment did not show a statistically significant difference compared with either PBS or AdlucRGD. However, it demonstrated that the oncolytic effect of Ad5-Δ24RGD is maintained (Ad5-Δ24RGD *versus* PBS, $P < 0.01$; Ad5-Δ24RGD *versus* Ad5-Δ24, $P < 0.05$). These variations observed between high-dose and low-dose experiments suggest that a threshold dose over 10^7 viral particles of Ad5-Δ24 is required

to obtain an oncolytic effect in tumor nodules (Fig. 4B). To confirm that the CRAds were present in the tumor tissue, we used immunofluorescence to detect the virus hexon in tumor samples collected after the low-dose experiment (35 days postinjection). Ad5-Δ24RGD was present in the tumor nodules, as was Ad5-Δ24 to a lesser extent. PBS- and Ad5lucRGD-treated nodules showed no hexon signal (Fig. 4C). These results corroborated the observation that the partial reduction of tumor mass was attributable to virus replication and that the RGD modification of the fiber knob conferred infectivity and oncolysis advantage to a CRAd *in vivo*.

DISCUSSION

CRAds are novel and promising agents for cancer therapy. However, their efficacy is predicated on efficient tumor infection, specific replication, and lateral spread. The deficiency of CAR in a variety of tumor targets is a limitation to adenovirus infection. In a previous report, we demonstrated that the insertion of an RGD motif into the HI loop of the fiber knob of nonreplicative adenoviruses enhances tumor infection (16, 17). This proves that CAR-independent entry represents a viable way to circumvent CAR deficiency in some tumor types.

In this report, we have demonstrated that the genetic introduction of an RGD sequence in the fiber of a CRAd, such as previously characterized Ad5-Δ24 (18), allows CAR-independent infection that leads to the enhancement of viral propagation and oncolytic effect *in vitro* and *in vivo*. The increased initial virus entry into the cells rendered by the RGD modification results in earlier detection and augmented yields of encapsidated DNA of Ad5-Δ24RGD compared with the unmodified Ad5-Δ24 (Fig. 2). Because this tropism modification is not anticipated to alter fundamental aspects of the viral replication cycle, this effect was likely attributable to the infectivity enhancement allowed by delivering the virus through CAR-independent pathways. Subsequently, we studied the oncolytic potency of CRAds in two cell lines and concluded that Ad5-Δ24RGD potency is higher than that of the unmodified virus. Although the XTT assay was not sensitive enough to demonstrate the lytic effect of Ad5-Δ24 compared with the nonreplicative Ad5lucRGD, the crystal violet showed early comet-like cytopathic areas in Ad5-Δ24-treated A549 and LNCaP cells, which indicated the presence of an incipient lytic effect, whereas Ad5lucRGD-treated cells were intact (Fig. 3A). The less notable difference between Ad5-Δ24RGD and Ad5-Δ24 seen in LNCaP cells is explained by the absence of the $\alpha_V\beta_3$ integrins (25), compensated by the presence of other types of RGD-binding integrins ($\alpha_3\beta_1$ and $\alpha_5\beta_1$; Ref. 26) that were rapidly saturated (Fig. 3).

Our ultimate goal was to demonstrate the superior oncolytic effect of Ad5-Δ24RGD in an *in vivo* model. To this end, A549 cells xenografted in nude mice were treated with single, high-dose (10^9 viral particles), i.t. injections of Ad5lucRGD, Ad5-Δ24, Ad5-Δ24RGD, or PBS, and the results showed that both CRAds (modified and unmodified) yielded similar oncolysis (Fig. 4A). However, when a 100-fold lower dose (10^7 viral particles) was administered, it became clear that the oncolytic effect of Ad5-Δ24RGD was higher than that of Ad5-Δ24 ($P < 0.05$; Fig. 4B). Furthermore, we were able to correlate the observed oncolytic effect with the presence of virus progeny in the tumor samples by immunofluorescent detection of adenoviral hexon. Hexon was not detected in PBS- (not shown) and Ad5lucRGD-treated nodules (Fig. 4C, a), whereas it was detected throughout the tumors treated with CRAds. The comparison between the two CRAds showed that fluorescence in Ad5-Δ24RGD-treated tumors was stronger than the one observed in Ad5-Δ24-treated tumors (Fig. 4C, b and c, respectively). The lack of fluorescent staining in tumors treated with the nonreplicative control Ad5lucRGD indicates that the detected hexon belongs to the viral progeny of Ad5-Δ24 and Ad5-Δ24RGD, and not to the initial inoculum. As regards the high divergence of the volumes of PBS- and Ad5lucRGD-treated tumors, factors such as highly heterogeneous cell replication rates and hypoxic and necrotic areas are known to affect individual tumor volume after a critical size is reached. These differences have been noted previously when using oncolytic viruses (27, 28). Nevertheless, total resolution of the tumors in the s.c. xenograft model was seen only in some nodules treated with Ad5-Δ24RGD, which indicated that administration volume and schema adjustments, such as the ones suggested recently by Heise *et al.* (29), might be necessary to achieve complete oncolysis.

As presented here and elsewhere (30), the efficacy of replication-competent viruses used as oncolytic agents can improved at the level of infectivity. As other tumor-binding peptides are isolated (30, 31), modifications in addition to the RGD insertion can be considered as well. Of note, the RGD modification described here does not preclude the binding of the fiber to CAR, and the modified virus can enter the cells through α_V integrins and CAR. One approach to improve specific tumor infection/transduction would be the combination of CAR ablation and tumor-specific ligands to redirect the virus tropism. Recently, the adenovirus fiber amino acids crucial for CAR-binding abrogation and new tumor-selective peptides have been defined (15, 31, 32). This combination will generate truly targeted viruses, but the efficiency of their propagation will depend on the amount of the targeted receptor in the same way as the propagation of the unmodified virus depends on CAR.

ACKNOWLEDGMENTS

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APPENDIX B

Advances in Brief

Efficient Oncolysis by a Replicating Adenovirus (Ad) *in Vivo* Is Critically Dependent on Tumor Expression of Primary Ad Receptors[1]

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Abstract

Replicating adenoviruses (Ads) are designed to replicate in and destroy cancer cells, generating viral progeny that spread within the tumor. To address the importance of the primary cellular receptor for Ads, the coxsackievirus and Ad receptor (CAR), in permitting intratumoral spread of a replicating Ad, we have used a pair of tumor cell lines differing only in the expression of a primary receptor for Ad5. This novel system thus allowed the first direct evaluation of the relationship between the efficacy of a replicating Ad and the primary receptor levels of the host cell without the confounding influence of other variable cellular factors. We demonstrate that the absence of the primary cellular receptor on the tumor cells restricts the oncolytic potency of a replicating Ad both *in vitro* and *in vivo*. Based on these findings, it is apparent that the potential therapeutic advantages afforded by viral replication would be negated by poor intratumoral spread of the viral progeny due to the failure to infect neighboring tumor cells. Because a number of studies have reported that primary cancer cells express only low levels of CAR, our results suggest that strategies to redirect Ads to achieve CAR-independent infection will be necessary to realize the full potential of replicating Ads in the clinical setting.

Introduction

The utility of replication-defective Ad vectors for cancer gene therapy is restricted by their inability to infect every cell within a solid tumor mass (1). The realization of this limitation has led to the development of a novel class of anticancer agents, conditionally replicating Ads.[3] These agents are designed to selectively replicate in and destroy cancer cells, followed by the release of the viral progeny by the lysed cells (2). The relative specificity of viral replication in tumor *versus* normal cells will therefore play a major role in dictating the safety and efficacy of replicating Ads. To this end, strategies to restrict the replication of Ads to tumor cells have either involved placing the expression of viral genes, most commonly the E1A gene, under the control of tumor- or tissue-specific promoters, or have been based on the complete or partial deletion of viral genes required for replication in normal cells, but not in tumor cells (3, 4). However, the efficacy of replicating Ads as oncolytic agents will also be dependent

on the ability of the viral progeny to achieve lateral infection and thereby spread within the tumor (3, 4).

The first step in Ad infection is the high-affinity binding of the COOH-terminal knob domain of the fiber capsid protein (5, 6) to the primary cellular receptor, CAR (7, 8). Subsequent internalization of the virion by receptor-mediated endocytosis is potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base with secondary host cell receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (9, 10). A number of studies have reported that primary cancer cells express only low levels of CAR and are therefore poorly infected by Ads (11–13). In accordance with this observation, the efficacy of Ad-mediated cancer gene therapy has been limited in preclinical and clinical studies by the resistance of the CAR-deficient tumor cells to Ad infection (14, 15). Consequently, considerable attention is being focused on strategies to modify Ad vectors to achieve efficient, CAR-independent gene transfer (16).

Based on these findings with replication-defective Ad vectors, we hypothesized that a low level of CAR expression on tumor cells would also restrict the efficacy of replicating Ads. In this regard, not only would a deficiency of CAR limit the efficiency of infection by the initial viral inoculum, but, more importantly, the potential therapeutic advantages afforded by viral replication would be negated by poor intratumoral spread of the viral progeny due to the failure to infect neighboring tumor cells.

In this study, we have investigated the hypothesis that the oncolytic potency of replicating Ads could be restricted by poor dissemination of the viral progeny due to the inability to infect tumor cells expressing low levels of CAR. To address this issue, we have used a pair of tumor cell lines that differ only in the expression of a primary receptor for Ad5. This novel system thus allowed the first direct evaluation of the relationship between the efficacy of a replicating Ad and the primary receptor levels of the host cell without the confounding influence of other variable cellular factors. We demonstrate that a deficiency of the primary Ad receptor on the tumor cells restricts the oncolytic potency of a replicating Ad, both *in vitro* and *in vivo*. This suggests that the efficacy of replicating Ads could be improved by modifications that allow CAR-independent infection of target cancer cells.

Materials and Methods

Viruses. Ad300wt, a wild-type human Ad serotype 5, was obtained from the American Type Culture Collection (Manassas, VA). AdGFP, an *E1-/E3-*deleted replication-deficient Ad5 vector that expresses GFP under the control of the cytomegalovirus promoter, has been described previously (17). The wild-type Ad and the vector were propagated in the permissive 293 cell line and purified by two rounds of cesium chloride density centrifugation. To determine the viral particle concentration, the virus was diluted in 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.1% SDS and incubated at 56°C for 10 min, and the absorbance at 260 nm was measured. Under these conditions, an absorbance of 1 corresponds to 1.1×10^{12} particles/ml (18).

Cell Lines. Human U118 MG glioma cells were obtained from the American Type Culture Collection. U118 MG-AR cells (previously designated

U118 MG-Ad5KsFv.rec cells), which express an artificial primary receptor for Ad5, have been described previously (19). The cells were propagated at 37°C in a 5% CO_2 atmosphere in DMEM/Ham's F-12 supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. U118 MG-AR cells were maintained in 400 μg/ml G418. FCS was purchased from Life Technologies, Inc. (Grand Island, NY), and media and supplements were from Mediatech (Herndon, VA).

Ad DNA Replication. U118 MG and U118 MG-AR cells cultured in 6-well plates were infected with Ad300wt or AdGFP at an MOI of 0.1 viral particle/cell. The culture medium was supplemented with 1 μCi/ml BrdUrd (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Attached and detached cells were harvested 4, 6, and 8 days after infection, and encapsidated DNA was purified after precipitating unencapsidated DNA with spermine (20). The viral DNA was digested with *Xho*I and resolved on a 1% agarose gel. The incorporation of BrdUrd into the DNA as a result of viral replication was determined by a Southwestern blot using a mouse anti-BrdUrd primary antibody (DAKO Corp., Carpinteria, CA) followed by a HRP-conjugated rabbit antimouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and detection with a Western blot chemiluminescence reagent (New England Nuclear Life Science Products, Boston, MA).

Ad Yield Assay. U118 MG and U118 MG-AR cells cultured in 6-well plates were infected with Ad300wt or AdGFP at an MOI of 0.1 viral particles/cell. Eight days after infection, the cells and media were harvested, and the viral titer was determined by a plaque assay on 293 cells.

CPE Assay. U118 MG and U118 MG-AR cells cultured in 24-well plates were infected with Ad300wt or AdGFP at MOIs of 0.1 and 1 viral particle/cell. Eight days after infection, the cells were fixed and stained with crystal violet.

In Vitro Cytotoxicity Assay. U118 MG and U118 MG-AR cells cultured in 24-well plates were infected with Ad300wt or AdGFP at an MOI of 1 viral particle/cell. Eight days after infection, a commercial cell proliferation assay (Promega, Madison, WI) was used to measure cell survival according to the manufacturer's instructions.

Animal Experiments. U118 MG and U118 MG-AR tumor xenografts were established by s.c. injection of 5×10^6 cells into the flank of 8–10-week-old female athymic nude mice (*nu/nu*; National Cancer Center, Frederick, MD). On reaching 60–100 mm³, the tumor nodules were injected with 50 μl of PBS or with a single dose of 10^8 particles of AdGFP or 10^6 particles of Ad300wt in 50 μl of PBS (five mice/group). Bidimensional tumor measurements were taken twice a week with calipers, and the tumor volume was calculated using the simplified formula for a rotational ellipsoid: $0.5 \times$ length $\times$ width^2 (21). Animals were followed for 38 days, until the tumor burden in some of the control groups became excessive, and the mice were sacrificed. Experiments were performed in accordance with federal and institutional guidelines for animal care.

Statistical Methods. Descriptive statistics (mean and SD) on tumor volume (mm³) were calculated per day for each treatment group. The percentage change in volume was calculated for U118 MG tumors infected with Ad300wt compared with the control groups treated with AdGFP or PBS. A similar procedure was used to calculate the percentage change in the volume of U118 MG-AR tumors infected with Ad300wt compared to the two controls, AdGFP and PBS. The mean percentage change in tumor volumes was compared between U118 MG and U118 MG-AR cells infected with Ad300wt using one-way ANOVA and tests of repeated measures using SAS software (version 6.12; SAS Institute, Inc. Cary, NC). The mean tumor volumes were compared between U118 MG tumors treated with PBS and U118 MG tumors treated with AdGFP using the ANOVA (*t* test). Likewise, the mean tumor volumes were compared between U118 MG-AR tumors treated with PBS and U118 MG-AR tumors treated with AdGFP. Similar tests were performed to compare mean tumor volumes between controls and Ad300wt-treated tumors. $P < 0.05$ was considered statistically significant in all of the analyses.

Immunohistochemistry. The presence of Ad5 capsid proteins in U118 MG and U118 MG-AR tumor sections was determined by immunohistochemical analysis using polyclonal rabbit anti-Ad5 antiserum (Cocalico, Reamstown, PA) as the primary antibody with an HRP-conjugated goat antirabbit secondary antibody (Jackson ImmunoResearch Laboratories). Diaminobenzidine (Sigma, St. Louis, MO) was used as the chromogenic substrate.

Results

To evaluate the hypothesis that the oncolytic potency of replicating Ads correlates with their ability to achieve intratumoral spread based on lateral infection of tumor cells by the viral progeny, we used a pair of cell lines that differ only in the expression of a primary receptor for Ad5. Human U118 MG glioma cells are refractory to Ad5 infection due to a paucity of CAR, although they express the α_v integrins necessary for virus internalization (12). We have previously generated a derivative cell line, designated U118 MG-AR, which is sensitive to Ad5 infection due to the expression on the cell surface of an artificial primary Ad5 receptor, in which the extracellular domain is derived from a single-chain antibody with specificity for the Ad5 knob (19). In preliminary experiments, we confirmed that a replication-defective Ad5 vector expressing GFP, AdGFP, was able to infect U118 MG-AR cells, although it failed to infect the parental U118 MG cells, which lack a primary receptor for Ad (data not shown).

Monolayers of U118 MG and U118 MG-AR cells maintained in medium supplemented with BrdUrd were infected at an MOI of 0.1 viral particle/cell with a wild-type, replicating Ad5, Ad300wt, or with a replication-defective Ad5 vector, AdGFP, as a control. Encapsidated Ad DNA was isolated from equivalent numbers of cells at various times after infection, digested with *Xho*I, and subjected to Southwestern blot analysis using an anti-BrdUrd antibody. As shown in Fig. 1, Ad DNA could not be detected 6 or 8 days after infection of U118 MG or U118 MG-AR cells with the replication-defective Ad5 vector AdGFP, although a very small amount of newly synthesized DNA was present at 4 days. In contrast, newly synthesized Ad DNA could be purified from cells infected with the wild-type Ad, with a significantly greater amount of encapsidated DNA present at all time points in U118 MG-AR cells compared with U118-MG cells. Thus, more Ad DNA was synthesized in the cells that express a primary Ad receptor and can therefore be infected by the replicating Ad.

We next sought to investigate whether the increased synthesis of viral DNA by the replicating Ad in cells expressing a primary cellular receptor for Ad5 would lead to an increase in the production of infectious viral progeny. To this end, monolayers of U118 MG and U118 MG-AR cells were infected with Ad300wt or AdGFP at an MOI of 0.1 viral particle/cell. Eight days after infection, the cells and media were harvested, and the viral titer was determined by a plaque assay on 293 cells. As expected, no infectious virus was produced in cells infected with AdGFP, a replication-deficient vector. Infection of primary receptor-negative U118 MG cells with Ad300wt resulted in
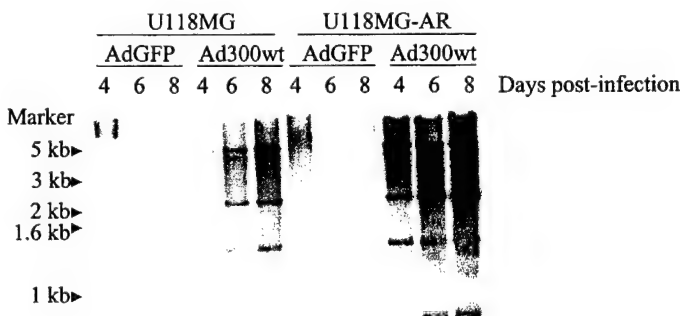
Fig. 1. Southwestern blot analysis of Ad DNA synthesis. Monolayers of U118 MG and U118 MG-AR cells maintained in medium supplemented with BrdUrd were infected with AdGFP or Ad300wt at an MOI of 0.1 viral particle/cell. Encapsidated Ad DNA was isolated from equivalent numbers of cells at the indicated times after infection, digested with *Xho*I, and subjected to Southwestern blot analysis using an anti-BrdUrd antibody. A significantly greater amount of DNA was synthesized by the replicating Ad in U118 MG-AR cells compared to U118-MG cells. Representative results are shown.

A

MOI	AdGFP		Ad300wt	
	0.1	1	0.1	1

U118MG

U118MG-AR

B

Fig. 2. *A*, CPE assay. Monolayers of U118 MG and U118 MG-AR cells were infected with AdGFP or Ad300wt at the indicated MOI. Eight days after infection, the CPE was monitored by staining the viable cells with crystal violet. The replicating Ad did not kill the parental U118 MG cells but caused extensive CPE in the U118 MG-AR cells.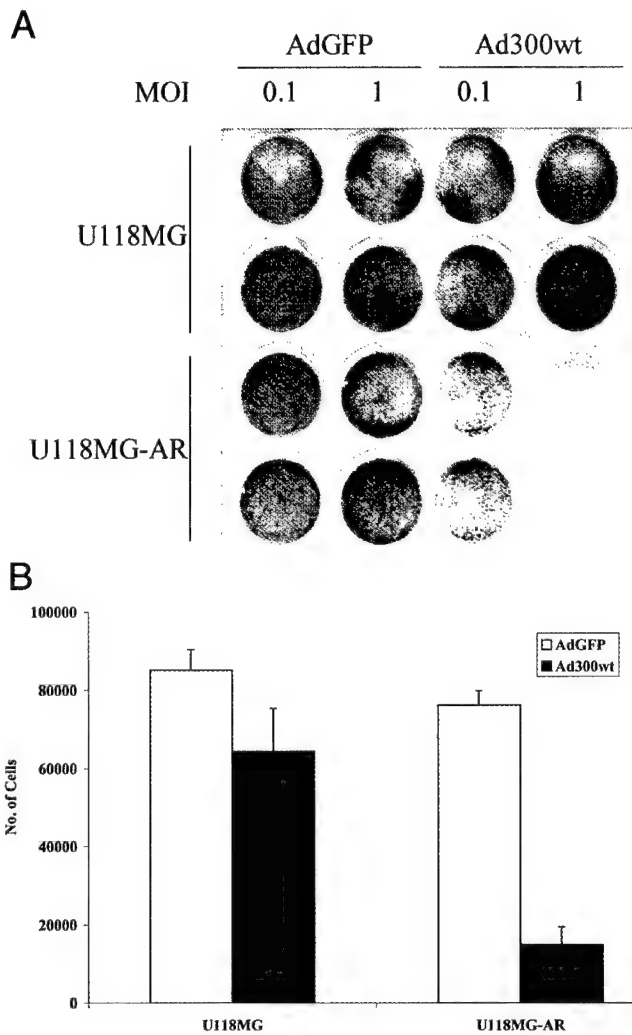 Representative results are shown. *B*, cell viability assay. Monolayers of U118 MG and U118 MG-AR cells were infected with AdGFP or Ad300wt at a MOI of 1 viral particle/cell. Eight days after infection, a cell proliferation assay was performed to count viable cells. The replicating Ad did not kill the parental U118 MG cells but caused a significant reduction in survival of the U118 MG-AR cells. Results are the mean of triplicate experiments.

4.0×10^7 plaque-forming units/ml, whereas the viral yield in U118 MG-AR cells was more than 57 times greater (2.3×10^9 plaque-forming units/ml). Thus, more Ad virions were produced in the cells that express a primary Ad receptor and can therefore be infected by the replicating Ad.

We then examined whether the increased yield of replicating viruses in cells expressing a primary cellular receptor for Ad5 was the result of the enhanced spread of the viral progeny through the monolayer during the several viral life cycles of the 8-day experimental period. Monolayers of U118 MG and U118 MG-AR cells were infected with Ad300wt or AdGFP at an MOI of 0.1 or 1 viral particles/cell. Eight days after infection, the CPE was monitored by staining the viable cells with crystal violet. As shown in Fig. 2A, the replication-defective AdGFP failed to lyse either cell line. Although the replicating virus did not kill the parental U118 MG cells, it caused extensive CPE in the U118 MG-AR cells, which were almost completely lysed at an MOI of 1 viral particle/cell. This indicates that the primary Ad receptor on the U118 MG-AR cells permitted lateral

infection by the replicating Ad, allowing the viral progeny to spread efficiently throughout the monolayer. This finding was confirmed by a quantitative assay in which viable cells were counted (Fig. 2B). Hence, the absence of the primary Ad receptor on the U118 MG cancer cells significantly reduced the oncolytic potency of the replicating Ad *in vitro*.

We next wished to determine whether the absence of the primary Ad receptor from cells in a solid tumor mass would restrict the ability of a replicating Ad to achieve lateral infection and spread within the tumor. Athymic nude mice bearing s.c. U118 MG or U118 MG-AR xenografts on the flank were injected intratumorally with a single dose of 10^6 particles of Ad300wt or with 10^8 particles of AdGFP or PBS alone as controls (five mice/group). Tumor growth kinetics are shown in Fig. 3. We observed that U118 MG tumors treated with PBS grew at a faster rate than U118 MG-AR tumors treated with PBS ($P = 0.0001$). Treatment of U118 MG tumors with the replication-defective AdGFP vector did not cause a significant reduction in volume compared to treatment with PBS ($P = 0.085$; Fig. 3A). Similarly, there was no significant difference in the size of U118 MG-AR tumors treated with either PBS or AdGFP ($P = 0.314$; Fig. 3B). Hence, intratumoral injection of the replication-defective vector

A

B
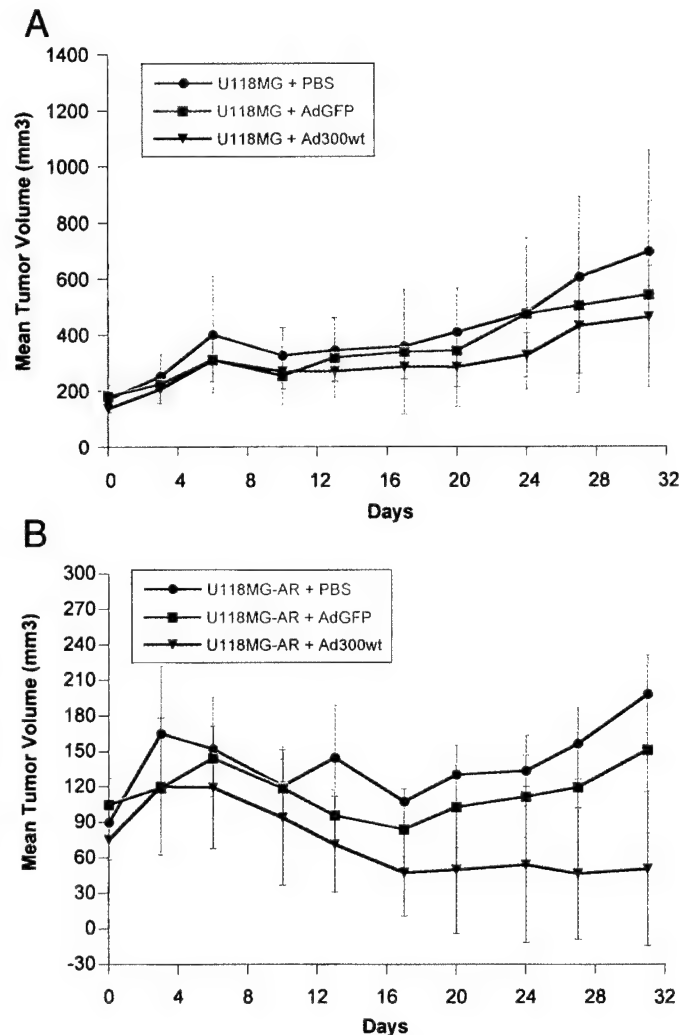
Fig. 3. Growth kinetics of s.c. U118 MG (*A*) and U118 MG-AR (*B*) tumors in athymic nude mice. Tumors were injected with a single dose of 10^8 particles AdGFP (*green squares*), 10^6 particles of Ad300wt (*blue triangles*), or with PBS (*red circles*). Data points represent the mean ± SE of the tumor size in each group at the indicated time points ($n = 5$). The oncolytic potency of the replicating Ad was significantly greater in the U118 MG-AR tumors than in the U118 MG tumors.

A.

B.

U118MG / AdGFP

U118MG / Ad300wt

C.

D.

U118MG-AR / AdGFP

U118MG-AR / Ad300wt

Fig. 4. Immunohistochemical staining of Ad5 capsid proteins in U118 MG and U118 MG-AR tumor xenografts treated with AdGFP or Ad300wt. Tumors were excised from nude mice 35 days after treatment. Tumor sections were subjected to immunohistochemistry using rabbit anti-Ad5 antiserum as the primary antibody with an HRP-conjugated goat antirabbit secondary antibody. Diaminobenzidine was used as the chromogenic substrate. Cells containing Ad5 capsid proteins are stained *brown*. *A*: cell, U118 MG; virus, AdGFP. *B*: cell, U118 MG; virus, Ad300wt. *C*: cell, U118 MG-AR; virus, AdGFP. *D*: cell, U118 MG-AR; virus, Ad300wt, with numerous adjacent cells stained positively for Ad5 capsid proteins, indicating the intratumoral spread of the replicating Ad.

did not affect tumor growth kinetics. Before statistical analysis of the effects of the replicating virus, it was necessary to adjust for the differential growth rates of the U118 MG and U118 MG-AR tumors in the control treatment groups. Ad300wt caused a significant reduction in the size of U118 MG-AR tumors compared to U118 MG tumors ($P = 0.0007$ when adjusted for treatment with AdGFP). Whereas U118 MG tumors treated with Ad300wt grew at rates comparable to the control groups ($P = 0.1119$ *versus* AdGFP), U118 MG-AR tumors injected with Ad300wt actually decreased in size ($P = 0.0001$ *versus* AdGFP), with complete tumor regression being observed in three of the five mice. Thus, the oncolytic potency of the replicating Ad was significantly greater in the solid tumors expressing a primary Ad receptor.

To confirm that the reduction in size of the U118 MG-AR tumors was due to intratumoral spread of Ad300wt, tumor sections were analyzed for Ad capsid proteins. Immunohistochemical staining with rabbit anti-Ad5 antiserum indicated that Ad300wt replicated and was disseminated throughout the U118 MG-AR tumors (Fig. 4). No staining was seen in U118 MG tumors injected with Ad300wt or in either U118 MG or U118 MG-AR tumors treated with AdGFP, the replication-defective Ad5 vector. Thus, the enhanced oncolytic potency of the replicating Ad in the solid tumors expressing a primary Ad receptor was due to increased intratumoral spread of the virus.

Discussion

A major limitation of cancer gene therapy strategies is the inability of replication-defective Ad vectors to disseminate throughout a solid tumor. A number of groups are seeking to address this problem by using Ads that replicate in cancer cells, thereby lysing the cells and releasing viral progeny that spread to neighboring cells. The potency of this novel class of anticancer agents will therefore depend on the efficiency of dissemination of the virus throughout the tumor.

In this study, we have investigated the hypothesis that the efficacy of replicating Ads could be restricted by poor dissemination of the viral progeny due to the inability to infect tumor cells expressing low levels of the primary Ad receptor. To address this issue, we have used a pair of tumor cell lines that differ only in the expression of a primary receptor for Ad5. We demonstrated that the oncolytic potency of a replicating Ad was significantly greater in the receptor-positive cell line, both in monolayers of cells *in vitro* and in solid tumors *in vivo*. Moreover, the greater efficacy of the replicating Ad in the receptor-positive tumors was due to increased spread of the virus. Therefore, we have shown that it is necessary for a replicating Ad to achieve efficient lateral infection of the tumor cells to realize its full potential as an anticancer agent. This novel model system thus allowed the first direct evaluation of the relationship between the efficacy of a replicating Ad and the primary receptor levels of the host cell without the confounding influence of other variable cellular factors.

A number of studies have reported that primary cancer cells from human patients express only low levels of the primary Ad receptor, CAR, and are therefore poorly infected by Ads (11–13). Based on our results, this suggests that the efficacy of replicating viruses dependent on CAR-mediated infection pathways will be restricted in the clinical setting. In accordance with this, Phase I and II clinical trials in which patients with recurrent squamous cell carcinoma of the head and neck received direct intratumoral injection of a replicating Ad, ONYX-015, resulted in clinical benefit in less than 15% of cases (22, 23). Only when combined with standard chemotherapy did this oncolytic Ad cause an objective response (at least a 50% reduction in tumor size) in 19 of 30 cases, with 8 complete responses (24).

The CAR deficiency of primary human cancer cells suggests that the efficacy of replicating Ads could be improved by modifying the viruses to allow efficient infection via a CAR-independent pathway. In this regard, Shinoura *et al.* (25) have reported that the potency of

a replicating Ad in glioma cell lines *in vitro* and *in vivo* could be improved by the addition of a stretch of 20 lysine residues to the COOH-terminal of the fiber protein, allowing the virus to bind to cellular heparan sulfate receptors. Similarly, Suzuki *et al.* (26) have shown that the efficacy of a replicating Ad can be enhanced by incorporating an RGD peptide motif into the fiber protein, permitting the virus to bind to α_v integrins. Each of these strategies to enhance the infectivity, and hence the potency, of replicating Ads resulted in expanded viral tropism: the viruses retained the ability to recognize the native primary Ad receptor, CAR, which is expressed by normal cells. Therefore, modifications to the fiber protein that both introduce a tumor cell-specific targeting motif and ablate recognition of CAR would simultaneously improve both the efficacy and safety of replicating viruses by permitting efficient, CAR-independent infection of tumor cells while preventing infection of normal cells (16). This would complement other strategies to restrict the replication of Ads to tumor cells, either by placing the expression of viral genes, most commonly the E1A gene, under the control of tumor- or tissue-specific promoters or by the complete or partial deletion of viral genes required for replication in normal cells, but not in tumor cells.

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APPENDIX C

Advances in Brief

Midkine Promoter-based Adenoviral Vector Gene Delivery for Pediatric Solid Tumors[1]

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Abstract

It is important to develop a system to express therapeutic genes in tumor cells with sufficient selectivity for cancer gene therapy. Midkine (MK) is a newly identified heparin-binding growth factor that is transiently expressed in the early stages of retinoic acid-induced differentiation of embryonal carcinoma cells. It has been reported that many human malignant tumors express high levels of MK mRNA or protein. However, no MK expression is detected in human or mouse liver. These interesting features of MK led us to examine the MK promoter as a candidate for tumor-specific gene expression. We thus developed new recombinant adenoviral (Ad) vectors containing either luciferase reporter gene (AdMKLuc) or herpes simplex thymidine kinase gene (AdMKTK) under the control of the human MK promoter. AdMKLuc achieved relatively high activity in Wilms' tumor (G-401) and neuroblastoma (SK-N-SH) cell lines. In addition, AdMKTK induced marked cell death in response to ganciclovir (GCV) in these same lines. Conversely, very low activity of the MK promoter was observed in mouse liver *in vivo* compared with the cytomegalovirus promoter. Importantly, AdMKTK + GCV did not induce liver toxicity, whereas substantial toxicity was seen with AdCMVTK + GCV treatment. On the basis of these findings, we conclude that the MK promoter is a candidate tumor-specific promoter for Wilms' tumor or neuroblastoma.

Introduction

The liver is the predominant site of Ad[3] vector localization after systemic administration (1) and as a consequence is at risk when Ad vectors containing suicide genes ectopically localize to this site. In this regard, ectopic expression of HSV-tk has been shown to cause substantial hepatic toxicity and morbidity in animal models (2, 3). These results indicate that the restriction of *tk* gene expression to tumor cells is critical to the safe application of the HSV-tk/GCV system. Thus, a promoter with both tumor specificity and minimal transcriptional activity in hepatocytes would be ideal for cancer gene therapy. Whereas a number of promoters have been explored in the context of cancer gene therapy, few exhibit this optimal profile of inductivity and specificity.

MK is a heparin-binding, growth/differentiation factor that is in-

duced by retinoic acid in embryonal carcinoma cells and is expressed temporally during the mid-gestational period of mouse embryogenesis (4). MK induces mitogenic activity in fibroblasts, has neurotropic activity (5), and promotes survival of retinal cells in constant light-induced retinal degeneration (6). It has been reported recently that many types of malignant tumors highly express MK (7–9), especially Wilms' tumors (8) and advanced neuroblastomas (9). Although inactivation of the Wilms' tumor suppressor gene (*WT1*) has been documented as one of the reasons that Wilms' tumors overexpress MK protein (10), the reason for MK expression observed in other tumors, including neuroblastomas, remains under investigation. The human MK promoter region contains an AP1 site, a DR5-type retinoic acid-responsive element, and consensus sequences for *WT1* (10, 11). Human MK and mouse MK are highly conserved, not only in cDNA structure but also in genomic organization and in discrete segments in the 5' upstream region (11); however, tissue distribution of MK is slightly different; in normal human tissues, MK is expressed moderately in the small intestine and weakly expressed in the lung, colon, and thyroid. In contrast, in mouse normal tissues, MK is expressed moderately in the kidney and weakly in the brain, but importantly, MK expression is not observed in mouse or human liver (8, 12). On this basis, the MK promoter is a potential candidate promoter for having the desirable features of high activity in tumor and low activity in the liver. In addition, the MK promoter shows strong activity when transfected into the Wilms' tumor G-401 cell line (10). We report herein the usefulness of MK promoter as a tumor-specific promoter for an Ad vector-based cancer gene therapy approach. A 2.3-kb upstream sequence of human *MK* gene (11), containing the promoter region, was inserted into an Ad vector and used to drive expression of either the luciferase reporter or HSV-*tk* genes. These vectors were then assessed *in vitro* and *in vivo* for activity and tumor specificity of transgene expression.

Materials and Methods

Cells and Cell Culture. The Wilms' tumor G-401, neuroblastoma SK-N-SH, colon cancer LS174T, and Burkitt's lymphoma Daudi cell lines were purchased from the American Type Culture Collection (Manassas, VA). G-401 cells were cultured in McCoy 5A medium containing 10% FCS. SK-N-SH and LS174T cells were maintained in MEM with 10% FCS and 1% nonessential amino acid (Mediatech/Cellgro). Daudi cells were cultured in RPMI 1640 with 10% FCS. All media and FCS using this study were purchased from Mediatech/Cellgro (VA).

RNA Preparation and Northern Blots. The MK cDNA probe corresponding to nucleotides 75–562, as reported by Tsutsui *et al.* (8), was used for this study. Total cellular RNA was extracted from 10^7 cells using an RNeasy kit (Qiagen). Twenty μg of total RNA were denatured with formaldehyde, electrophoresed though a denaturing 1% agarose gel, and transferred to a Hybond nylon membrane (Amersham). Membranes were hybridized with a ^{32}P-labeled probe prepared using Ready To Go DNA labeling beads (Pharmacia Biotech) at 42°C for 4 h. Membranes were then washed twice with 2×

SSC for 10 min and twice with 0.2× SSC at 56°C for 30 min. Membranes were exposed to BioMax film (Kodak) at −80°C with an intensifying screen for 2 days.

Viruses and Viral Techniques. The recombinant Ad vectors AdMKLuc and AdMKTK, encoding firefly luciferase and HSV-tk, respectively, under the control of human MK promoter containing 27 bp of exon 1 and 2285 bp of the 5′ flanking region of the human *MK* gene, were constructed using the "Ad-Easy" method reported previously (13). Briefly, the MK promoter and either a luciferase gene (pGL3 basic vector; Promega) or *tk* gene (14) was inserted into a multicloning site in pShuttle vector. The resultant plasmid was linearized with *Pme*I digestion and subsequently cotransfected into *Escherichia coli* BJ5183 with pAdEasy-1 Ad backbone plasmid. After confirming recombination, the recombinants of interest were linearized with *Pac*I digestion and transfected into 293 cells to generate AdMKLuc or AdMKTK. The recombinant adenoviruses were propagated in 293 cells and purified by double CsCl density centrifugation. Virus titers were determined by plaque assay in 293 cells. We also used Ad vectors containing luciferase or *tk* gene under the control of CMV enhancer/promoter as control vectors.

In Vitro **Gene Transfer.** Tumor cells were plated in 24-well plates in triplicate at the concentration of 50,000/well 1 day prior to gene transfer. After overnight culture, the cells were infected with AdMKLuc or AdCMVLuc at a MOI of 50 pfu in medium containing 2% FCS for 1 h. The infecting medium was then replaced with complete medium. The infected cells were harvested and treated with 100 μl of lysis buffer 2 days after infection. A luciferase assay (Luciferase Assay System; Promega) and a luminometer (Lumat; Wallac, Inc.) were used for the evaluation of luciferase activities of Ad-infected cells. Luciferase activities were normalized by the protein concentration in cell lysate (Bio-Rad DC Protein Assay kit).

GCV Sensitivity of Cell Lines Transduced with AdMKTK or AdCMVTK at Four Different MOIs. Tumor cells were plated in 96-well plates in triplicate at a density of 3000/well. After overnight culture, cells were infected with AdMKTK or AdCMVTK at MOIs of 0, 10, 50, and 100 for 5 h. The viral infection was followed by medium replacement including various concentrations of GCV ranging from 0 to 1000 μM. The number of surviving cells was determined by MTT assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega) after 5 days exposure of GCV. The MTT color development was measured and analyzed by an automated E max spectrophotometric plate reader (Molecular Device Corp., Sunnyvale, CA).

In Vivo **Gene Transfer.** For determination of luciferase gene expression in mouse organs, C57/BL6 (Charles Rivers) mice received 1×10^9 pfu of AdMKLuc or AdCMVLuc by tail vein injection. Three days later, livers, kidneys, lungs, and spleens were harvested to measure the luciferase gene expression.

s.c. tumors were induced by injection of 10^7 G-401 cells into the flank of the congenitally athymic nude mice (Charles Rivers). When tumor formation was confirmed (4–6 mm in diameter), AdMKLuc (2×10^8 pfu) or AdCMVLuc (1×10^8 pfu) were injected into the tumor. Two days later, mice were sacrificed, and tumors were resected, placed in the polypropylene tubes, and immediately frozen in ethanol/dry ice. Frozen tissues were ground to a fine powder using a pestle and mortar immersed in an ethanol/dry ice bath. Tissue powder was lysed using a tissue lysis buffer (Promega), and then luciferase activity was determined using a luciferase assay system kit (Promega). The luciferase activity was normalized by protein concentration in the tissue lysate.

To investigate the hepatic toxicity induced by the HSV-tk/GCV system, C57/BL6 mice received 1×10^9 pfu of AdMKTK or AdCMVTK. The next day, i.p. GCV treatment was started (50 mg/kg, twice daily). After 7 days of continuous GCV administration, mice were sacrificed, and blood samples were taken to assess AST, ALT, total bilirubin, and LDH. Livers were fixed in 10% buffered formalin overnight and then processed into paraffin blocks, and 4-μm sections were cut, deparaffinized, and stained with H&E.

Results

In Vitro **Luciferase Inducement by the Recombinant Adenovirus.** *MK* gene expression was examined in several human cell lines by Northern blotting (Fig. 1A). As reported previously (8, 9), G-401 Wilms' tumor and SK-N-SH neuroblastoma cells highly expressed MK mRNA. In contrast, no MK expression was observed in the LS174T colon cancer cell line. Although Daudi lymphoma cells were
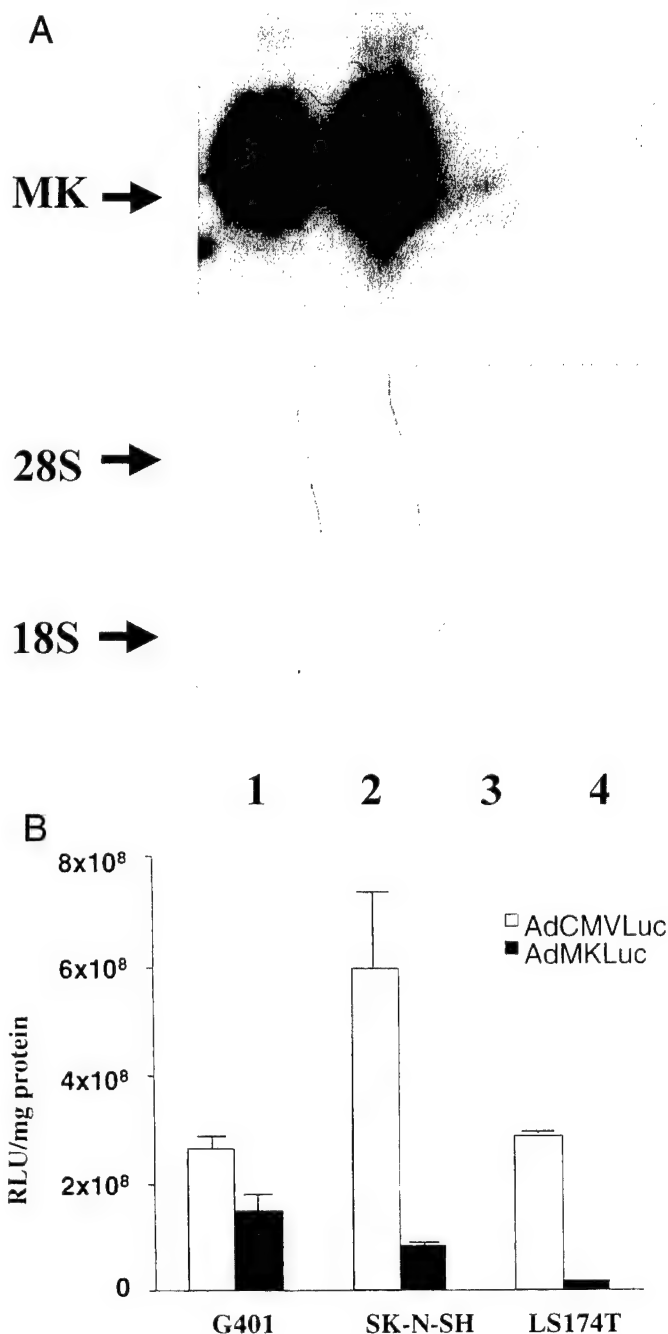

Fig. 1. *A*, MK mRNA expression in cell lines. The *arrow* of the *upper panel* shows MK mRNA. The *lower lane* shows 18S and 28S rRNA stained by ethidium bromide. Each lane contains 20 μg of total RNA. *Lane 1*, G-401; *Lane 2*, SK-N-SH; *Lane 3*, LS174T; *Lane 4*, Daudi cell line. *B*, luciferase inducement in cell lines by AdMKLuc or AdCMVLuc. The means of the luciferase inducement by AdCMVLuc are 2.6×10^8 RLU/mg protein in G-401, 5.9×10^8 RLU/mg protein in SK-N-SH, and 2.9×10^8 RLU/mg protein in LS174T. On the other hand, those by AdMKLuc are 1.5×10^8 RLU/mg protein in G-401, 8.5×10^7 RLU/mg protein in SK-N-SH, and 1.7×10^7 RLU/mg protein in LS174T.

used as a negative control of MK expression, this lymphoma cell line was not appropriate for the assessment of Ad gene infection, because these cells are refractory to infection by Ad. We have already confirmed that the MK promoter, extending from 27 bp of exon 1 to 2285 bp of the 5′ flanking region of the human *MK* gene, showed relatively high activity in G-401 cells in a plasmid context (10). We thus investigated whether the MK promoter in the adenoviral context would manifest high transcriptional efficiency in MK-positive cells. In a reporter gene experiment with firefly luciferase, tumor cells were
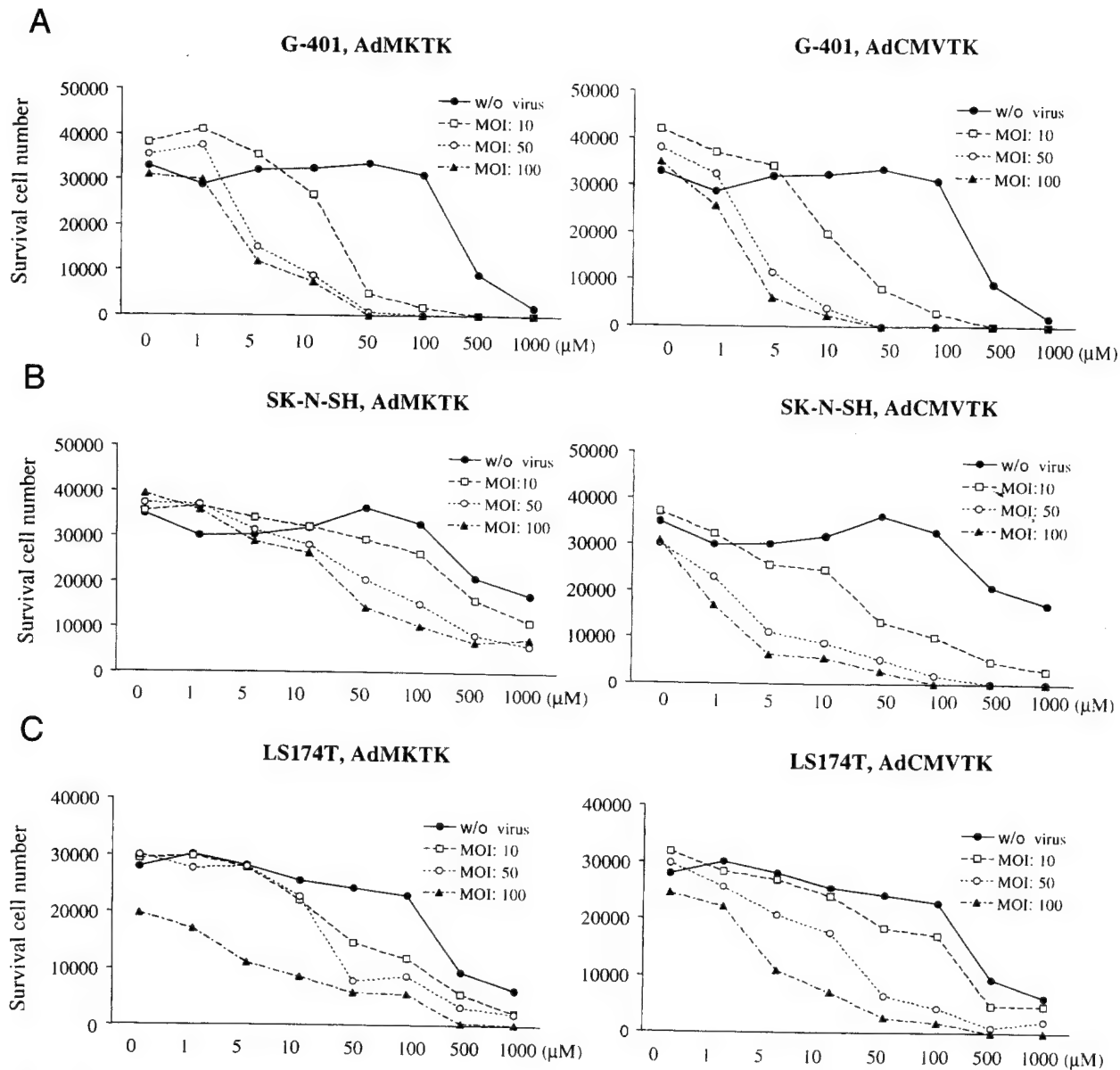
Fig. 2. GCV sensitivity of tumor cell lines transduced with AdMKTK or AdCMVTK at four different MOIs. The tumor cells were infected with AdMKTK or AdCMVTK at MOIs of 0, 10, 50, and 100. GCV was applied at concentrations ranging from 0 to 1000 μM, and 5 days later the number of surviving cells was determined by MTT assay.

infected with AdMKLuc or AdCMVLuc at a MOI of 50 (Fig. 1B). In the G-401 cell line, luciferase activity induced by AdMKLuc showed comparatively high activity, approximately half the activity induced by AdCMVLuc infection. In the SK-N-SH cell line, the luciferase activity induced by AdMKLuc infection was less, ~15% of that induced by AdCMVLuc. In comparison with tumor-specific promoters published previously (15, 16), this level was high. On the other hand, luciferase inducement by AdMKLuc was low in LS174T compared with AdCMVLuc. These results indicate that the MK promoter activity in the Ad context reflects the MK expression level of the cell lines.

AdMKTK Confers GCV Sensitivity to Various Tumor Cell Lines. To determine whether Ad-mediated infection with the MK-TK gene would render various cell lines sensitive to cell killing by GCV, the tumor cells were infected with AdMKTK or AdCMVTK at MOIs of 0, 10, 50, and 100. The recombinant Ad infection was followed by 5 days of GCV exposure at concentrations ranging from 0 to 1000 μM, and then the number of surviving cells was determined by MTT assay.

AdMKTK successfully induced GCV sensitivity in the G-401 line (Fig. 2A). Using AdMKTK at the dose of 50 MOI, IC_{50}s (the GCV concentration at which 50% cell survival is seen) are 4.5, 67, and 31 μM in G-401, SK-N-SH, and LS174T, respectively. Whereas for AdCMVTK at the same dose, the IC_{50}s are 3.6, 3.7, and 19 μM in G-401, SK-N-SH, and LS174T, respectively. In the G-401 cells, the effectiveness of this vector was comparable with AdCMVTK, as reflected by the similar IC_{50}s. AdMKTK/GCV also achieved cell killing in the SK-N-SH line, although this was less effective than the AdCMVTK/GCV combination. Although the luciferase study showed that the MK promoter activity was relatively low in the LS174T cells, this level of activity was sufficient to induce cell killing with Ad-MKTK/GCV. This may be a reflection of the sensitivity of the cell line to this particular suicide gene approach.

MK Promoter in Ad Context Has Low Activity in the Liver *in Vivo*. A key limitation to the use of HSV-tk/GCV approach for cancer therapy is the potential for toxicity to non-target organs. Because the Ad has particular tropism for the liver and because hepatic toxicity
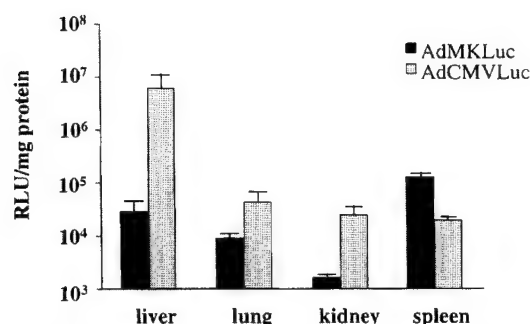
Fig. 3. Tissue specificity of the MK promoter in adenoviral context. Mice received 1×10^9 pfu of AdMKLuc or AdCMVLuc via tail vein injection (six per group). Two days after virus injection, mice were sacrificed to obtain the organ samples. The means of luciferase expression after AdMKLuc administration are 2.9×10^4 RLU/mg protein in liver, 9.0×10^3 RLU/mg protein in lung, 1.7×10^3 RLU/mg protein in kidney, and 1.3×10^5 RLU/mg protein in spleen. On the other hand, those after AdCMVLuc administration are 5.9×10^6 RLU/mg protein in liver, 4.3×10^4 RLU/mg protein in lung, 2.4×10^4 RLU/mg protein in kidney, and 1.9×10^4 RLU/mg protein in spleen. Bars, SE.

can be induced by HSV-tk/GCV, we were especially interested to determine whether the MK promoter would have low activity in the liver *in vivo*. Thus, AdMKLuc or AdCMVLuc (as a positive control) was injected i.v. into mice, and then the level of transgene expression at day 2 was determined (Fig. 3). In this assay, transgene expression induced by the MK promoter was a mean 200-fold less than that seen with the CMV promoter. This is consistent with previous studies that have shown no MK expression in mouse or human liver. Interestingly, the AdMKLuc-induced luciferase expression in the spleen was actually higher than that of AdCMVLuc. Although the possible reasons for this are speculative, it does indicate that the low luciferase results seen in the liver with AdMKLuc were not attributable to a fundamental problem with the efficacy of this vector *in vivo per se*. These results thus indicate the key property of MK promoter fidelity in the context of the Ad vector used *in vivo*.

MK Promoter Activation in the Ad Context Had High Activity in Tumor *in Vivo*. As a correlate to the finding that the MK promoter had very low activity in the liver, we investigated the ability of AdMKLuc to transduce G-401 tumors *in vivo*. To assess this, tumors were implanted s.c. in nude mice and then injected with either AdCMVLuc or AdMKLuc (Fig. 4). Because the initial *in vitro* study had shown that luciferase expression using AdMKLuc was ~50% of that seen with AdCMVLuc (Fig. 1B), we used doses of 2×10^8 pfu or 1×10^8 pfu of AdMKLuc or AdCMVLuc, respectively. These studies confirmed the functionality of the MK promoter in MK-positive cells *in vivo*. Thus, these studies showed that the MK promoter in the context of an Ad vector possesses the two critical elements for consideration for use for cancer gene therapy, high-level expression in tumor and low-level expression in the liver.

GCV Administration Combined with AdMKTK Does Not Cause the Liver Dysfunction. As a final step, we investigated the effect on the liver of AdMKTK/GCV or AdCMVTK/GCV treatment. Mice were administered AdMKTK or AdCMVTK by tail vein injection, followed by either no further treatment or 7 days treatment with GCV. All of the mice that received 1×10^9 pfu of AdCMVTK, and GCV administration manifested general weakening, weight loss, or reduced activity after 7 days of GCV injection. On the other hand, mice in other groups showed no abnormal features. The mice in this study were sacrificed after 7 days of GCV administration, and blood samples and livers were harvested to evaluate the liver dysfunctions. A prominent elevation of serum AST, ALT, total bilirubin, and LDH was observed in the AdCMVTK/GCV-treated mice as compared with

the other groups (Fig. 5A). In addition, the livers in this group appeared macroscopically yellowish, suggestive of fatty change, whereas the livers of mice that received AdMKTK/GCV appeared virtually normal (Fig. 5B). Microscopically, the livers of the mice that received AdMKTK with or without GCV and of the animals that received GCV only did not show significant pathology; however, they did show some evidence of mild extramedullary hematopoiesis. In contrast, the AdCMVTK with GCV showed severe changes with microvesicular fatty change, individual cell necrosis, and acute inflammation, as well as extramedullary hematopoiesis. Minor inflammatory and fatty changes were noted in the AdCMVTK animals that did not receive GCV. Thus, these results demonstrate a key finding with respect to the use of the MK promoter for suicide gene therapy, *i.e.*, prevention of hepatic toxicity, and indicate the potential benefits of using this promoter for cancer gene therapy.

Discussion

In this study, we show for the first time that the MK promoter has tissue-specific fidelity in the Ad backbone. These findings indicate that this promoter may be an ideal candidate for tumor-specific suicide gene therapy. In this regard, the very low level of expression in hepatocytes should help to mitigate against the liver toxicity reported for the AdHSV-tk/GCV approach. AdMKTK did not cause liver dysfunction, despite GCV administration. This finding, coupled with the relatively high levels of expression seen in tumors *in vivo*, suggest this novel approach will have a significantly enhanced therapeutic window compared with the use of viral promoters such as CMV. The findings reported herein are important for several reasons. Although many tumor-specific promoters have been proposed for use in gene therapy vectors, these agents typically exhibit levels of activity much lower than viral promoters. This fact has lead to the development of amplification strategies to enhance the efficiency of a specific but weak promoter (15, 16). Thus, the finding that the AdMK viruses were capable of driving transgene expression at a level comparable with the CMV promoter is especially significant. Furthermore, many candidate tumor-specific promoters have been seen to have their specificity undermined when placed in the Ad backbone (17). This effect is poorly defined but may be attributable to *cis*- or *trans*-acting enhancing elements in the genome of the Ad vector. This finding has necessitated the use of insulator sequences to improve the fidelity of some promoters such as Erb B2 (18). In the current study, however, we demonstrate that the MK promoter retains its specificity in the Ad context. Levels of activity correlated with levels of MK expression in cell lines, and most importantly, the expression in the liver, where MK is not normally expressed, was very low. In addition, MK is expressed in certain tumor types for which current therapies are
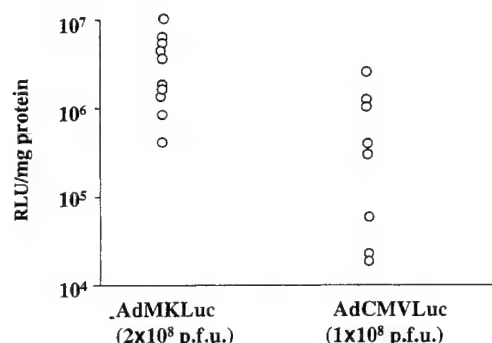

Fig. 4. Luciferase expression in s.c. tumor injected with either AdMKLuc or AdCMVLuc. AdMKLuc (2×10^8 pfu) was injected into 10 s.c. G-401 tumors and AdCMVLuc (1×10^8 pfu) into 8 s.c. tumors. The median of luciferase expression by AdMKLuc is 4.0×10^6 RLU/mg protein and that by AdCMVLuc is 4.1×10^5 RLU/mg protein.
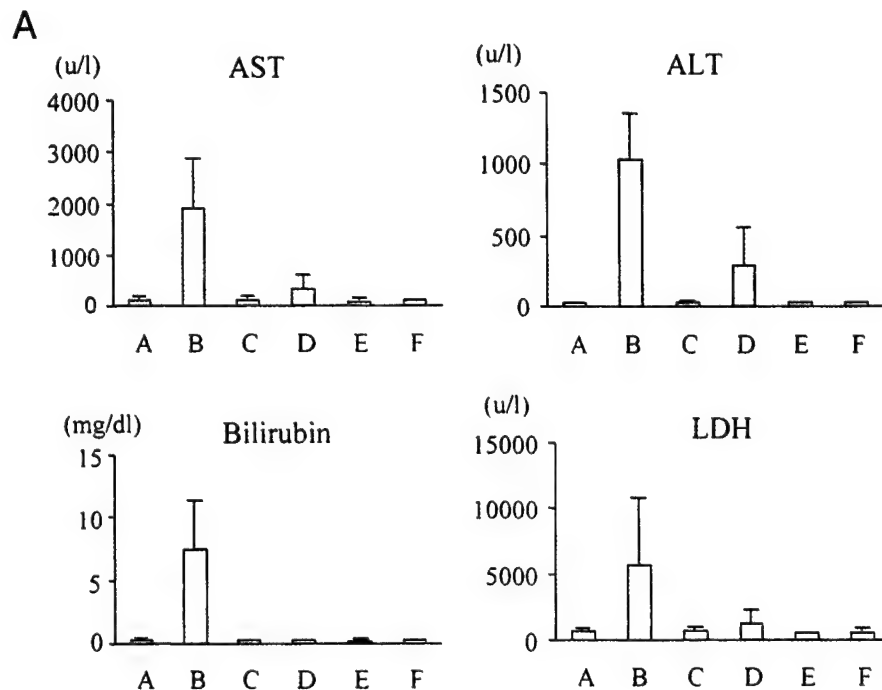
Fig. 5. A, serum levels of AST, ALT, total bilirubin, and LDH after AdHSVtk/GCV treatment. Mice received 1×10^9 pfu of AdMKTK or AdCMVTK via tail injection. Blood samples were obtained after a 7-day GCV treatment ($2 \times$ 50 mg/kg per day). Group A, AdMKTK + GCV treatment ($n = 4$); group B, AdCMVTK + GCV treatment ($n = 5$); group C, AdMKTK ($n = 4$); group D, AdCMVTK ($n = 4$); group E, GCV treatment ($n = 4$); group F, no treatment ($n = 4$). B, macroscopic findings of the livers of mice treated with either AdMKTK/GCV (group A) or AdCMVTK/GCV (group B).
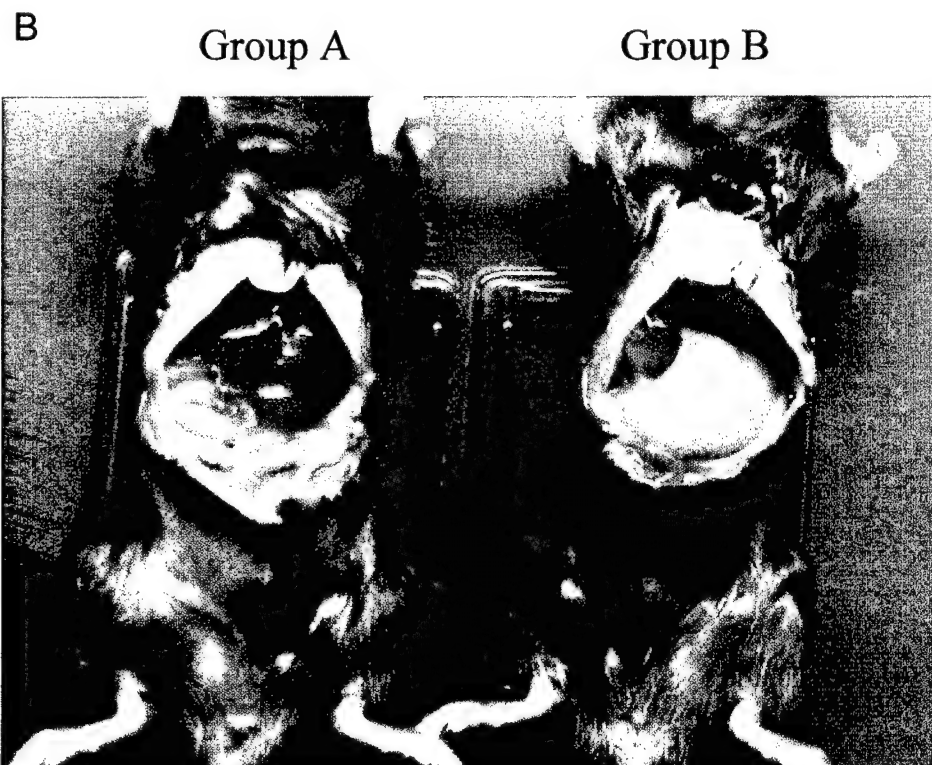
inadequate. In this regard, G-401 cells are properly regarded as a model for malignant rhabdoid tumor of the kidney (MRTK; Ref. 19), a disease that shows poor response to conventional therapies (20). Thus, the high level of activity of AdMKTK in this line suggests that this approach may offer new therapeutic options for this disease. Furthermore, the treatment of advanced neuroblastomas, ordinarily occurring in older children and manifesting bone metastasis or aggressive local spread, is one of the crucial problems in pediatric oncology. In this context as well, various approaches, including high-dose chemotherapy combined with bone marrow transplantation, have not proven to be satisfactory. In this

regard, Nakagawara et al. (9) reported that MK was expressed at high levels in almost all neuroblastomas, and aggressive neuroblastoma seemed to have a relatively increased amount of MK. Thus, the finding that AdMKTK has efficacy in a neuroblastoma line is significant with respect to the potential of applying gene therapy approaches, as we have proposed here.

In summary, we believe the MK promoter is a unique candidate tumor-specific promoter by virtue of its very low hepatic activity and toxicity, high tumor activity, and fidelity in the Ad vector. This new approach may have utility not only for Wilms' tumors, neuroblastomas, but other MK-positive neoplasms (7, 8).

Acknowledgments

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APPENDIX D

Characterization of the Cyclooxygenase-2 Promoter in an Adenoviral Vector and Its Application for the Mitigation of Toxicity in Suicide Gene Therapy of Gastrointestinal Cancers

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ABSTRACT

Background and Aim: The application of herpes simplex virus thymidine kinase-based adenoviral molecular chemotherapy for systemic malignant disease has been limited by ectopic expression of the transgene within the liver. The aim of this study was to mitigate the toxicity of this treatment using the promoter of cyclooxygenase-2, whose expression is virtually undetectable in liver but is detected in many gastrointestinal cancers. *Methods:* Two different length of cyclooxygenase-2 promoters were incorporated into adenoviral vectors. The promoters were characterized with luciferase expression vectors. The specific cytocidal effect and *in vivo* toxicity were analyzed with thymidine kinase expression vectors. *Results:* The specificity of the cyclooxygenase-2 promoter was well preserved in the adenoviral vector. *In vivo*, the cox-2 promoter (-1432/+59) showed very little activity in the liver but accomplished high activity, comparable to the cytomegalovirus promoter, in cyclooxygenase-2 positive subcutaneous tumors. The cyclooxygenase-2 promoter driven thymidine kinase-expressing vectors showed a cytocidal effect specifically in cyclooxygenase-2 positive cells. When mice were treated with the thymidine kinase-expressing vector and ganciclovir, the cyclooxygenase-2 promoter successfully mitigated the fatal hepatotoxicity, which was observed with the cytomegalovirus-promoter driven vector. *Conclusions:* The cyclooxygenase-2 promoter successfully mitigated the adverse effects of adenoviral suicide gene therapy by minimizing transgene expression in the liver.

2

INTRODUCTION

Advanced gastrointestinal cancers, especially those associated with distant metastatic disease, exhibit a very high mortality rate despite available therapies [1-2]. A variety of experimental therapeutic strategies have been explored for advanced gastrointestinal cancers including gene-based applications using suicide/toxin gene methods, such as herpes simplex virus thymidine kinase (HSV-tk) [3-5]. As adenoviral vectors can achieve efficient *in situ* gene delivery to tumors [6], they have been widely employed as a means to achieve the requisite intratumoral transgene expression for toxin gene approaches in locoregional gastrointestinal cancer models [7-8].

By contrast, derivation of adenoviral suicide gene therapy to the disseminated disease of gastrointestinal cancers has not currently been feasible. Because of the hepatotropism of adenovirus, systemically administered adenoviral vector localizes principally to the liver [9-10] and adenoviral suicide gene therapy of intrahepatic tumor leads to severe liver dysfunction [11-12]. Due to this vector physiology, the direct application of adenoviral vectors-based toxin gene approaches for disseminated or intrahepatic lesions has been hampered.

To overcome this obstacle, a number of strategies have been proposed to restrict the toxin gene expression to the tumor. The approach of transductional targeting seeks to alter vector tropism at the level of receptor interaction to achieve tumor-selective infection [13-14]. Alternatively, transcriptional targeting is based upon selective expression of toxin genes in tumor targets. Such an approach must thus employ a transcriptional control region with a selective "tumor on" phenotype. In addition, candidate promoters are also required to exhibit a "liver off" phenotype for mitigation of hepatotoxicity upon systemic delivery or the treatment of intrahepatic lesions.

Cyclooxygenase-2 (cox-2) is an inducible isoform of the cyclooxygenase family and is virtually undetectable in most tissues under physiological conditions [15-16]. On the other hand, cox-2 is closely linked to carcinogenesis and progression of colon cancers ([17-18]); 85 % of colon cancers, 45 % of benign colon polyps and the majority of gastric cancers have been reported to show increased expression of cox-2 [17-19-26]. This tumor "on" liver "off" expression profile of cox-2 suggests the potential utility of this promoter for mitigating the toxicity caused by ectopic transgene expression.

In this study, we explored the cox-2 promoter as a candidate transcriptional control region for gastrointestinal tumor targets. The properties of the cox-2 promoter in adenoviral vectors were analyzed *in vitro* and *in vivo*. These studies establish that the cox-2 promoter exhibits the key properties for the feasibility of the adenoviral gene therapy for gastrointestinal cancers.

MATERIAL AND METHODS
Cell Culture and Animals

The Caco-2, WiDr and LS174T colon cancer cell lines (American Type Culture Collection, ATCC, HTB-37, CCL-218 and CL 188, respectively; Manassas, VA) were grown in Minimum Essential Medium with non-essential amino acids (Mediatech, Herndon, VA). The MKN28, MKN45 and KATO-3 gastric cancer cell lines (Japanese Collection of Research Bioresources, JCRB, JCRB0253, JCRB0254, JCRB0611, respectively; Tokyo, Japan) and lung cancer cell lines A549 (ATCC CCL-185) and H358 (ATCC CRL-5807) were grown in RPMI 1640 (Mediatech). The HepG2 (ATCC HB-8065), HuH-6 clone5 (JCRB0401), HuH-7 (JCRB0403) liver cancer cell lines and 293 adenoviral transformed human embryonic kidney cell line (ATCC CRL-1573) were maintained with Dulbecco's Modified Eagles Medium. All the media were supplemented with 10% fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were incubated at 37 °C and 5% CO_2.

Female C57BL/6 mice (Charles River, Wilmington, MA) and female Nu/Nu athymic nude mice (Frederick Cancer Research, Frederick, MD) (6-8 week old) were used for *in vivo* experiments. All animals received humane care based on guidelines set by the American Veterinary Association. All the experimental protocols involving live animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Analysis of Cox-2 RNA Status

The cox-2 RNA status of cell lines was analyzed by reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was extracted from semi-confluent cell cultures on 10 cm dishes using the RNeasy mini RNA extraction kit (QIAGEN, Valencia, CA) as described by the manufacturer. Five hundred nanograms of total RNA were reverse transcribed with oligo-dT primer and amplified by polymerase chain reaction using the GeneAMP RNA PCR Kit (Perkin Elmer, Branchburg, NJ) as described by the manufacturer except the extension time was 60 sec. The primers used for the analysis of cox-2 RNA were cox-2 sense (5' ggtctggtgcctggtctgatgatg 3') and cox-2 antisense (5' gtcctttcaaggagaatggtgc 3'). As a control, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA was also analyzed in the same way using GAPDH sense (5' caactacatggtttacatgttccaa 3') and GAPDH antisense (5' gccagtggactccacgacgt 3') primers.

Adenovirus Vectors

The recombinant adenoviral vectors that express firefly luciferase and HSV-tk respectively were constructed through homologous recombination in *Escherichia coli* using the AdEasy system ([27])(Figure 1). All vectors used in these experiments had the transgene cassettes in the E1 deleted region of adenoviral vector backbone. Two different lengths of promoters derived from phPES2 (provided by Drs. Inoue and Tanabe at National Cardiovascular Center Research Institute, Japan [28-29]) were placed in front of each transgene for selective expression: one is cox-2L (-1432/+59, the whole 5' control region of phPES2) and the other is cox-2M (-883/+59, SacI-HindIII fragment of cox-2L). Though three major control regions of the cox-2 promoter (binding sites of NF-κB, NFIL-6 and CRE) exist within 300 base pairs from transcription initiation site, the longer control regions were used to achieve as much fidelity as possible. The resultant vectors expressing luciferase were designated Adcox-2L Luc and Adcox-2M Luc, and those expressing HSV-tk were designated Adcox-2L TK and Adcox-2M TK, respectively. The luciferase gene and Simian virus 40 poly adenylation signal were from pGL3 Basic (Promega, Madison WI). As control vectors that expressed the transgene ubiquitously, luciferase and HSV-tk expression vectors with the cytomegalovirus (CMV) immediate early promoter (derived from plasmid pCEP4, Invitrogen, Carlsbad, CA) instead of the cox-2 promoter were also constructed and named AdCMV Luc and AdCMV TK, respectively.

The infectivity enhanced version of luciferase expression vectors, which confer coxsackie-adenovirus receptor independent infection *via* the integrin binding of the RGD motif in the adenovirus fiber HI-loop [30-31], were constructed with cox-2L and cox-2M promoters and plasmid pVK503C [31] the resultant vectors were named as RGDcox-2L Luc and RGDcox-2M Luc, respectively.

The viruses were propagated in the adenovirus packaging cell line, 293, and purified by double CsCl density gradient centrifugation, followed by dialysis against phosphate-buffered saline with 10% glycerol. The vectors were titrated by plaque assay and stocked at –80°C until usage.

In vitro Analysis of Cox-2 Promoter

The activity of the cox-2 promoter in an adenovirus context was analyzed by infection of cells with luciferase expression vectors. One day after plating 50,000 cells per well on 24 well plate, cells were infected at a multiplicity of infection (MOI) of 50 plaque forming unit (pfu) per cell with Adcox2L Luc and Adcox2M Luc, respectively, in Dulbecco's Modified Eagles Medium with 5% fetal calf serum (infection medium) for one hour. Two hours later, the infection medium was replaced with the appropriate complete medium. After 48 hours of cultivation, the cells were lyzed with Cell Culture Lysis Buffer (Promega) and resultant lysates were analyzed with the Luciferase Assay System (Promega). The protein concentration was determined with the DC protein assay (Bio-Rad, Hercules, CA). For RGD-containing vectors, the same experiments were performed using RGDcox-2L Luc and RGDcox-2M Luc at an MOI of 10 pfu per cell. All the experiments were done in triplicate.

In vivo Analysis of Cox-2 Promoter

To analyze the luciferase gene expression in mouse organs, C57BL/6 mice received 10^9 pfu of Adcox2L Luc, Adcox-2M Luc or AdCMV Luc intravenously *via* the tail vein. Two days later, the livers, lungs, kidneys and spleens were harvested to measure luciferase expression.

For the analysis of subcutaneous tumors, 2×10^7 cultivated cells were inoculated subcutaneously into the flank of the congenitally athymic nude mice, and 10^9 pfu of Adcox2L Luc, Adcox2M Luc or AdCMV Luc were injected into the tumors when tumors of 6-8 mm diameter were formed. Two days later, the tumors were resected for luciferase analysis. All the organs and tumors were rapidly frozen on dry ice and stored at -80 °C until assayed. On the day of analysis, tissues were ground into fine powder with a pestle and mortar in an ethanol/dry ice bath. The tissue powders were lysed with Cell Culture Lysis Buffer (Promega) and, after three rounds of freezing and thawing followed by centrifugation, the recovered supernatants were analyzed for luciferase activity with the Luciferase Assay System (Promega). The protein concentration was determined with DC protein assay (Bio-Rad). All the experiments were done in triplicate.

In vitro Analysis of the Cytocidal Effect of HSV-tk Expression Vectors in Combination with GCV

Tumor cells were plated in 96 well plates at a density of 3,000 per well. The following day, the cells were infected for 5 hours with 100 µl of infection medium containing Adcox2L TK, Adcox2M TK and AdCMV TK, respectively. To compensate the relatively large infection volume, the infections were performed at MOI of 500. The infection mediums were then replaced with appropriate medium supplemented with various concentrations of ganciclovir (GCV). As a negative control, a green fluorescent protein expression vector (AdGFP) was used. After 5 days of incubation, the number of surviving cells was analyzed by the MTS method using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) and an automated E max spectrophotometric plate reader (Molecular Device Corp., Sunnyvale, CA) as described by the manufacturers. All the experiments were done in triplicate.

In vivo Analysis of the Toxicity of the Cox-2 Promoter Driven HSV Thymidine Kinase in Combination with GCV

To investigate the toxicity induced by the HSV-tk/GCV system, each group of ten C57BL/6 mice received a tail vein injection of AdCMV TK (10^9 pfu), Adcox2LTK (10^9 pfu) or phosphate-buffered saline on day 0. Five of each group received GCV (50mg/kg body weight) intraperitoneally twice daily from day 1 to day 5 and the others did not. The mice were sacrificed on day 6 and the blood samples were collected to analyze total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) at a veterinary laboratory (ANTECH Diagnostics, Farmingdale, NY). For histopathological analysis, the major organs were fixed with 10% buffered formaldehyde, paraffin-embedded and cut in to 4μm section, followed by deparaffinization and staining with hematoxylin and eosin.

RESULTS
Analysis of cox-2 RNA

The cox-2 RNA status of the cell lines used for this experiment was analyzed by RT-PCR (Figure 2). Because the primers were designed to have three introns between them, the signal detected at 723 bp represented complementary DNA that was reverse transcribed from RNA. In the liver cell lines (HuH6, Huh7 and HepG2), there was no band detected. Two gastric cancer cell lines (MKN28 and MKN45) and one lung cancer cell line (A549) showed a very strong signal and two colon cancer cell lines (LS174T and Caco-2) showed a moderate signal. On the other hand, one gastric cancer cell line (KATO-3) and a lung cancer cell line (H358) did not show a signal for cox-2 RNA.

In vitro analysis of the cox-2 promoter in an adenoviral construct

To analyze the function of the cox-2 promoter in an adenoviral construct, cell lines were infected with cox-2 promoter-driven luciferase expression vectors (Adcox2L Luc and Adcox2M Luc). The cox-2 positive cells expressed luciferase but cells that were considered to be negative for cox-2 expression in RT-PCR analysis also showed high luciferase activity. This was most remarkable in liver derived cells (Figure 3A and B). In case of using the vectors with RGD modification (RGDcox2L Luc and RGDcox2M Luc), which can infect cells in a coxsackie-adenovirus receptor-independent manner, the expression in the non-liver derived cells increased and, as a result, the relative expression in the liver derived cells became much lower than that in most of the cox-2 positive cell lines (Figure 3C and D). In comparison to the cox-2 L promoter, the cox-2 M promoter showed slightly higher activity, but the overall profiles of these two were almost similar.

In vivo analysis of the cox-2 promoter in an adenoviral construct: activity in major organs

After intravenous administration of Adcox2L Luc, Adcox2M Luc and AdCMV Luc, the activity of the cox-2 promoter in four major organs (liver, lung, kidney and spleen) was assessed by luminometric analysis (Figure 4). In the liver, the luciferase activity with Adcox2M Luc (89500 relative light units (RLU)/mg protein, n=3) was less than 1/400 of that with AdCMV Luc (41700000 RLU/mg protein, n=3). The activity with Adcox2L Luc, which had longer promoter sequence, (1320 RLU/mg protein, n=3) was even lower than that with Adcox2M Luc and less than 1/30000 of AdCMV Luc (Figure 4A). In contrast, the activities in the spleen with these three vectors were within the same order of magnitude (16200, 39300 and 61700 RLU/mg protein for Adcox2L Luc, Adcox2M Luc and AdCMV Luc, respectively, n=3) (Figure 4B). However, in the lung, each of the two vectors with cox-2 promoters showed two orders of magnitude lower activity than that seen with CMV promoter (Figure 4C). In the kidney, only Adcox2L Luc showed lower activity than that seen with others (Figure 4D).

In vivo analysis of cox-2 promoter in an adenoviral construct: activity in subcutaneous tumors

The activity of the cox-2 promoter in an adenoviral construct was compared with the CMV promoter in gastrointestinal cancer subcutaneous tumors. In subcutaneous tumor xenografts of two cox-2 strongly positive cell lines, MKN28 and MKN45, the activities of the cox-2L promoter were comparable to the CMV promoter (12900000 RLU/mg protein to 55100000 RLU/mg protein for MKN28, 1760000 RLU/mg protein to 3360000 RLU/mg protein for MKN45) (Figure 5A and 5B). In the case of a moderately cox-2 positive cell line, LS174T, the activity of the cox-2 promoter was detected but lower than that with CMV promoter (362000, 868000 and 73900000 RLU/mg protein with Adcox2L Luc, Adcox2M Luc and AdCMVLuc, respectively) (Figure 5C). Thus, adenoviral vector mediated delivery to subcutaneous tumors *in*

vivo could accomplish strong transgene expression in cox-2 positive cells at the level comparable to the CMV promoter.

Cytocidal effect of the cox-2 promoter driven HSV-tk expression vector in combination with GCV

The specificity of the cytocidal effects with the cox-2 promoter driven HSV-tk expression vectors was analyzed in cell lines. An obvious cytocidal effect comparable to AdCMV TK was observed with Adcox2M TK and Adcox2L TK not only in two cox-2 strongly positive cells (MKN28 and MKN45, Figure 6A and 6B) but also in the cox-2 moderately positive cell line (LS174T, Figure 6C). In contrast, in cox-2 negative cell line (Kato-3), a strong cytocidal effect was observed only with AdCMV TK (Figure 6D). These results clearly indicate that either length of cox-2 promoter (cox-2 L and cox-2 M) conferred selective cell killing in cox-2 positive cancer cells in the HSV-tk expressing adenoviral vector.

Mitigation of hepatotoxicity of adenoviral suicide gene therapy employing the cox-2 promoter

To investigate whether the cox-2 promoter could mitigate the hepatotoxicity of adenoviral suicide gene therapy, the toxicity of GCV after intravenous administration of the cox-2L and CMV promoter driven HSV-tk expression vectors was analyzed. In the mice treated with AdCMV TK and GCV, the activity and food intake began to decrease from the day three, and 2 of the 5 mice of this group died on day 6. Macroscopically, in all surviving mice of this group, the color of the liver appeared yellowish, suggestive of fatty change (Figure 7A). No remarkable abnormality was observed in other organs. Neither a decrease of activity nor macroscopic abnormality was observed in any other group including the group that received Adcox2LTK and GCV (figure 7B-7D).

Microscopically, in the group treated with AdCMV TK and GCV, most of the hepatocytes showed extensive microvesicular fatty changes. There was also swelling of the individual hepatocytes, scattered individual cells undergoing necrosis, and marked nuclear pleomorphism of hepatocytes. This group also had marked congestion of the hepatic parenchyma (Figure 8A). In the group with Adcox2L TK and GCV (Figure 8B), the tinctorial pattern of hepatocytes was similar to the pattern seen in the untreated control (Figure 8D). In this group, there were scattered individual necrotic cells and small areas of cellular dropout as well as focal areas of extramedullary hematopoiesis. In addition, this group showed scattered lymphocytes present in the hepatic parenchyma and the Kupffer cells were prominent. The livers of the group which received AdCMV TK without GCV (Figure 8C) were remarkable for the extensive nuclear pleomorphism of the hepatocytes. There was an increase in mitosis, individual cell necrosis and apoptotic bodies. Though there was focal acute and lymphocytic inflammation scattered throughout the hepatic parenchyma, many necrotic cells were not surrounded by inflammatory cells. Overall, the patterns seen in this group was consistent with increased hepatic cellular injury and death followed by hepatic regeneration. The livers of the groups without virus administration appeared almost normal regardless of GCV administration (Figure 8D).

No differences were observed in the histologic characteristics of the kidneys or the lungs among all groups. Spleens of groups which received AdCMV TK showed extensive extramedullary hematopoiesis and the demarcations between the white and red pulp were obscured. The group with Adcox2L TK and GCV showed some extramedullary hematopoiesis, although less extensive than that seen in the group with AdCMV TK.

In the analysis of the serum samples, an elevation of the total bilirubin level was observed only in the group with AdCMV TK and GCV (Figure 9A). In contrast, abnormality of ALT, AST and LDH appeared in the groups with AdCMV TK regardless of GCV administration (Figure 9B-D). Neither of the groups that received Adcox2L TK (with or without GCV) showed abnormality

compared with the untreated group. The group that received only GCV (without virus) did not show abnormality.

Taken together, the lethal hepatotoxicity with massive microvesicular fatty change and jaundice observed in AdCMV TK with GCV group was mitigated by substituting the CMV promoter with the cox-2 L promoter.

DISCUSSION

Adenoviral vectors have been widely used for cancer gene therapy [6]. However, in the context of suicide gene therapy, the expression of the effector gene in the liver due to vector hepatotropism is problematic as it can cause fatal liver dysfunction [32]. Especially in gastrointestinal cancers, where the most probable administration route is intravenous or transcatheter administration into the feeding artery of the tumor, the expression of a suicide gene in the liver is clearly one of the major obstacles to the clinical use of adenoviral vectors for suicide gene therapy. To employ transcriptional targeting to overcome this issue, a promoter that is inactive in the liver but strongly active in gastrointestinal cancer cells is required. In the present study, the cox-2 promoter, which is expressed in many gastrointestinal cancers [17] but not expressed in most of the organs under normal conditions [15], was incorporated into an adenoviral vector construct and characterized *in vitro* and *in vivo*, and its feasibility for mitigating liver toxicity in suicide gene therapy was studied.

When the cox-2 promoters were incorporated in adenoviral vectors with unmodified fiber, the activity profile of *in vitro* experiments did not correlate with the cox-2 RNA status but rather showed correlation with infectivity (data not sown), mainly due to the apparent high luciferase activity observed in liver cancer cell lines. There were two possible explanations for these results: one is that higher infection efficiency of liver cancer-derived cells caused this high activity in these cells, the other is that the incorporation of the cox-2 promoter into an adenoviral vector made the promoter "leaky" as has been observed for other promoters [6-33-35]. To study this point, the RGD motif was incorporated into the H-I loop of the fiber knob region [30]. When using this infectivity-enhanced vector, with which the variation of infection efficiency was minimized from 20 fold to 20% (data not shown), the overall results showed correlation not with the infectivity but with cox-2 RNA status. This clearly indicated that the cox-2 promoter was working properly in the adenoviral construct and also suggested that the reason for the unexpected results with the adenovirus with unmodified fibers was not the leakiness of the promoter, but the variation of the infection efficiency.

In the experiments of tail vein administration of luciferase-expressing adenoviral vectors, the luciferase activity in the liver with the cox-2 M promoter was less than 1/400 of that with the CMV promoter and, moreover, that with cox-2 L promoter was even lower, less than 1/30000 of CMV promoter. On the other hand, the activities in the spleen with these three promoters were within the same order of magnitude. These results indicated that the cox-2 promoters are not generally weak but negative in the liver specifically. The cox-2 promoters, especially the cox-2 L promoter, had remarkable reduction of the transgene expression in liver and were potentially applicable for avoiding the liver toxicity caused by undesired transgene expression at that site. Interestingly, although major positive control regions exist in the last 300bp of these promoters, the activity of the cox-2 L promoter in the liver was much lower than the cox-2 M and this remarkable difference did not exist in the analysis using cell lines. This might suggest that a transcription suppressor sequence functioning in the *in vivo* liver existed between -1432 and -883. When the promoter was analyzed in the subcutaneous tumors, the cox-2 L promoter in cox-2 positive cells (MKN28 and MKN45) was very strong and comparable to the CMV promoter. Because the CMV promoter has been recognized as one of the strongest promoters in an adenoviral context [6-36], it may be understood that the cox-2 promoter is a relatively strong promoter in cox-2 positive tumors.

When the cox-2 promoters were incorporated into the HSV-tk expression vectors, the cox-2 promoter-driven vectors (Adcox2M TK and Adcox2L TK) showed strong cytocidal effects selectively on cox-2 positive cells, unlike the vector with the CMV promoter that killed all the cell lines regardless of cox-2 status. This confirmed that the cox-2 promoter was strong and selective enough to kill cox-2 positive cells in suicide gene therapy with HSV-tk and GCV. At the same time, because it is reported that the specificity of some promoters may vary with distinctive

transgenes [37], it is important that selectivity of the promoter was confirmed with two different transgenes.

The *in vitro* and *in vivo* analyses indicated that the cox-2 L promoter was suitable for mitigation of the liver toxicity caused by the undesired transgene expression because its low activity in the liver and high activity in cox-2 positive tumors were well preserved in the adenoviral constructs. Thus, the *in vivo* toxicity of the cox-2 L promoter driven HSV-tk expression vector, in combination with GCV, was compared with that of the CMV promoter. When AdCMV TK was administered, GCV caused remarkable toxicity where 2 of the 5 mice of that group died at day 5. In this group, the liver of the surviving mice showed severe macroscopical and microscopical abnormality and the blood analysis showed hyperbilirubinemia and liver dysfunction. In contrast, in the group with the Adcox2LTK in combination with GCV administration, the liver was almost normal except for rare dropout of hepatocytes and extramedullary hematopoiesis. In addition, there was no significant abnormality in the blood analysis. This clearly indicated that the "liver off / tumor on" profile of the cox-2 promoter mitigated the fatal liver toxicity. Interestingly, even without GCV administration, the liver receiving AdCMV TK showed extensive damage without microvesicular fatty change. Because the histological localization of the liver damage in this group was independent from lymphocyte infiltration, it suggested that this liver damage was caused by direct toxicity of HSV-tk expression in the liver, independently from cellular immunity.

Looking for the method to overcome the safety issue, some tumor specific promoters have been employed to drive HSV-tk expression in adenoviral vectors. In the context of suicide gene therapy of Wilms' tumor and neuroblastoma, the midkine promoter is reported to be very beneficial for the mitigation of liver toxicity [38]. In the field of gastrointestinal cancers, though the carcino-embryonic antigen promoter is reported to show a useful profile in terms of low activity in the liver, the activity of this promoter on the positive cell lines is relatively low and required a 20 fold dose to obtain transgene expression of a comparable level to that of the CMV promoter [39]. Because the fatal toxicity in the dose escalation in a clinical study of ornithine transcarbamylase deficiency suggesting the possibility of toxicity of adenoviral vectors at high dose [40], such dose increase as a means to overcome the weakness of the promoter is not practical for clinical use. Thus, selective and strong promoters in gastrointestinal cancers are required to make gene therapy feasible and, in this context, the cox-2 promoter appears to be especially promising.

In the present study, we characterized the cox-2 promoter in an adenoviral constructs and showed it could reduce undesired transgene expression in the liver. In suicide gene therapy with an HSV-tk expressing adenoviral vector and GCV administration, this promoter mitigates the hepatotoxicity after systemic administration. This indicates that the cox-2 promoter can be very beneficial for gene therapy of intrahepatic lesions by transcatheter intraarterial administration or intratumoral injection, where the transgene expression in the background liver can cause safety problems [12]. To make gene therapy not only feasible but also clinically useful, strategies to ensure key safety endpoints are required. In this regard, cox-2 promoters are useful to reduce the adverse effect caused by the undesired transgene expression in the liver and have widespread applicability for many kinds of cox-2 positive cancers [41-44].

FIGURE LEGEND

Figure 1. Structure of the recombinant adenoviral vectors.

Adenoviral vectors were constructed by inserting the respective expression cassettes into the E1-deleted region of the E1- and E3-deleted backbone. Two different length of cox-2 promoters (cox-2 L: from -1432 to +59 and cox-2M: from -883 to +59) were placed 5' of the transgene to drive expression. The luciferase expression vectors with cox-2L and cox-2 M promoters were named Adcox2L Luc and Adcox2M Luc, respectively. The HSV-tk expressing vectors with cox-2L and cox-2 M promoters were named Adcox2L TK and Adcox2M TK, respectively. The Simian virus 40 polyadenylation signal was placed on the 3' of the transgene.

Figure 2. Analysis of cyclooxygenase-2 RNA.

The RNA of the cell line used in these experiments was analyzed by reverse transcription followed by polymerase chain reaction. Panel A: The signal for cyclooxygenase-2 RNA was detected at the position of 723 bp with cox-2 sense (5' ggtctggtgcctggtctgatgatg 3') and cox-2 antisense (5' gtcctttcaaggagaatggtgc 3') primers. Panel B: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA was detected with using GAPDH sense (5' caactacatggtttacatgttccaa 3') and GAPDH antisense (5' gccagtggactccacgacgt 3') primers.

Figure 3. Promoter property analysis of cell lines.

Each cell line was infected with cox-2 promoter driven luciferase expression vectors and luciferase activity was analyzed. The results were shown after standardization with protein concentrations. A and B are the results with cox-2 M and L promoter driven regular adenoviral vectors, respectively. C and D are those with the RGD modification.

Figure 4. Cox-2 promoter activity in major organs.

Luciferase activities were analyzed *in vivo* on four major organs (A: liver, B: spleen, C: lung and D: kidney) after tail vein administration of cox-2 or CMV promoter driven luciferase expression vectors. The luciferase activities are shown as RLU per mg protein.

Figure 5. The cox-2 promoter activity in subcutaneous tumors. The luciferase activities were analyzed in three subcutaneous tumors (A: MKN28, B: MKN45 and C: LS174T) after intratumoral injection of cox-2 or CMV promoter driven luciferase expression vectors. The luciferase activities are shown as RLU per mg protein.

Figure 6. The cytocidal effect of cox-2 promoter driven HSV-tk expression vectors.

Cells were infected with cox-2 promoter driven HSV-tk expressing vectors (Adcox2M TK and Adcox2M TK) and the number of surviving cells was analyzed using the MTS method after 5 days of treatment with various concentrations of GCV. AdCMV TK and AdGFP were used as positive and negative control vectors, respectively. (A: MKN28 (cox-2 positive), B: MKN45 (cox-2 positive), C: LS174T (cox-2 positive) and D: KATO3 (cox-2 negative))

Figure 7. Mitigation of toxicity of suicide gene therapy with the cox-2 L promoter: Macroscopic findings.

After systemic administration of HSV-tk expressing adenoviral vector (10^9 pfu) followed by 5 days of GCV (50mg/kg weight, twice a day, intraperitoneal), the major organs were macroscopically observed. (A: AdCMV TK with GCV, B: Adcox2L TK with GCV, C: AdCMV TK without GCV and D: no treatment)

Figure 8. Mitigation of toxicity of suicide gene therapy with cox-2 L promoter: Microscopic findings of the liver.

The major organs of the mice in each group were microscopically analyzed after H and E staining. The findings of the liver are shown in this figure. The open arrows indicate cells undergoing individual necrosis. Solid arrow indicates mitosis. (A: AdCMV TK with GCV, B: Adcox2L TK with GCV, C: AdCMV TK without GCV and D: no treatment, Original magnification x600)

Figure 9. Mitigation of toxicity of suicide gene therapy with the cox-2 L promoter: blood sample analysis.
The blood samples of each group were analyzed for ALT, AST, total bilirubin and LDH. (A: AdCMV TK with GCV, B: Adcox2L TK with GCV, C: no virus with GCV, D: AdCMV TK without GCV, E: Adcox2L TK without GCV, F: no treatment)
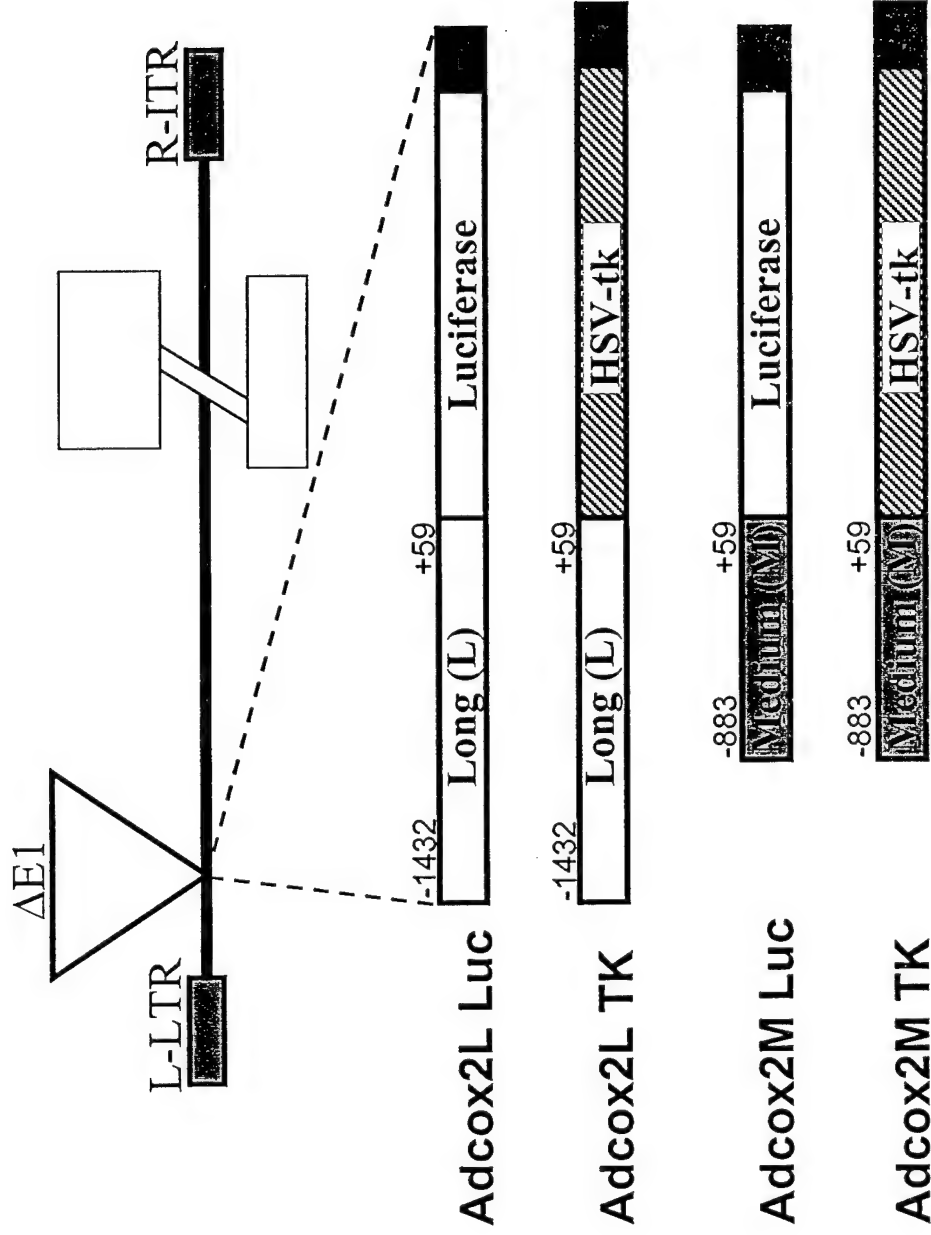
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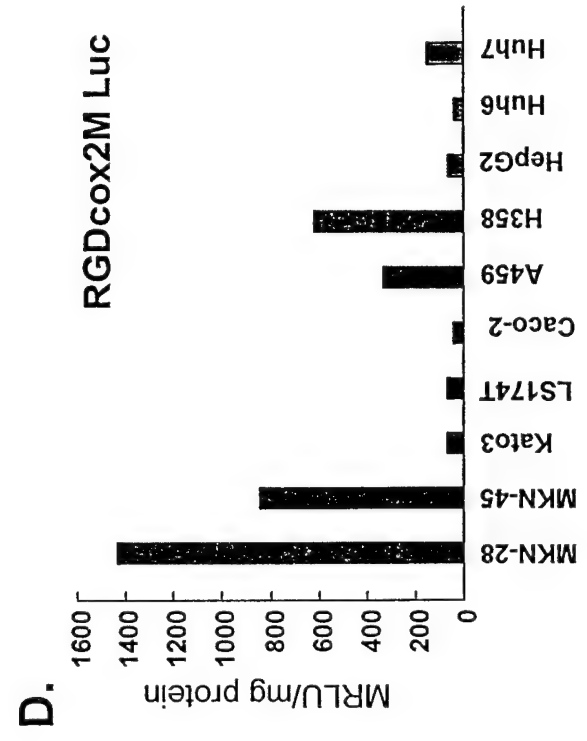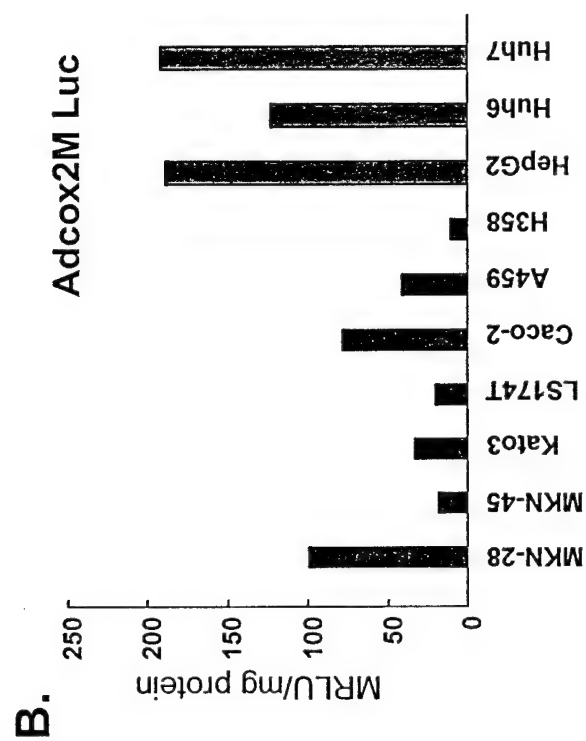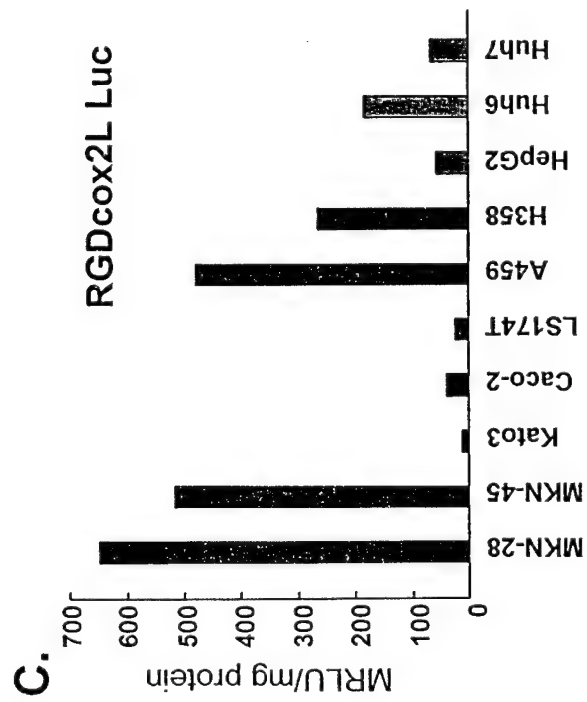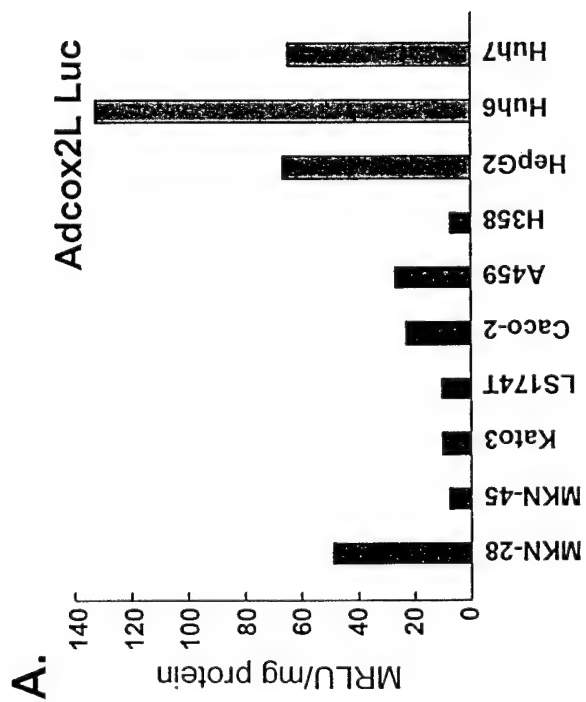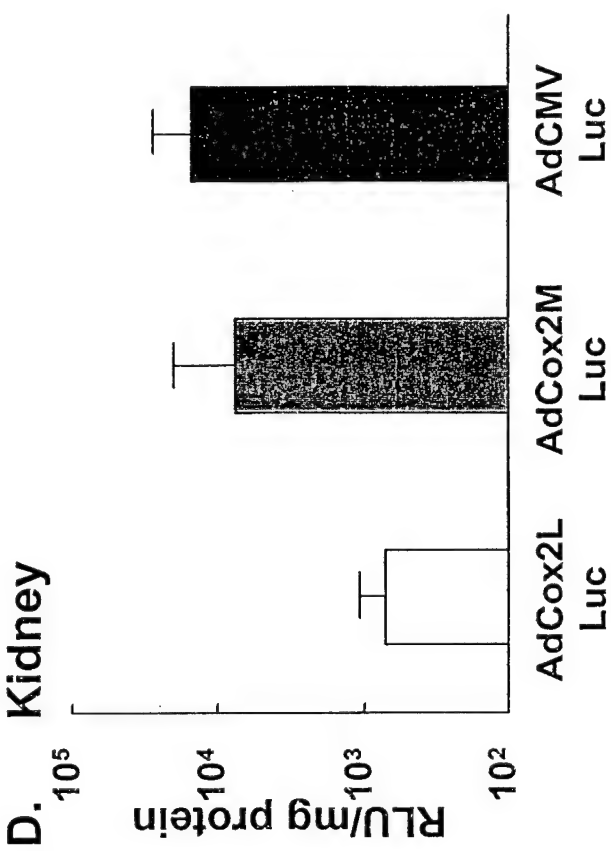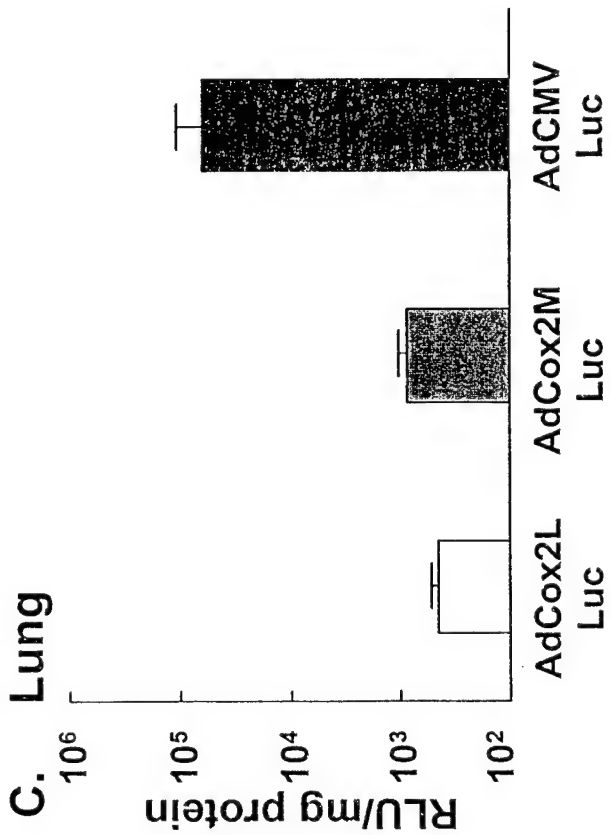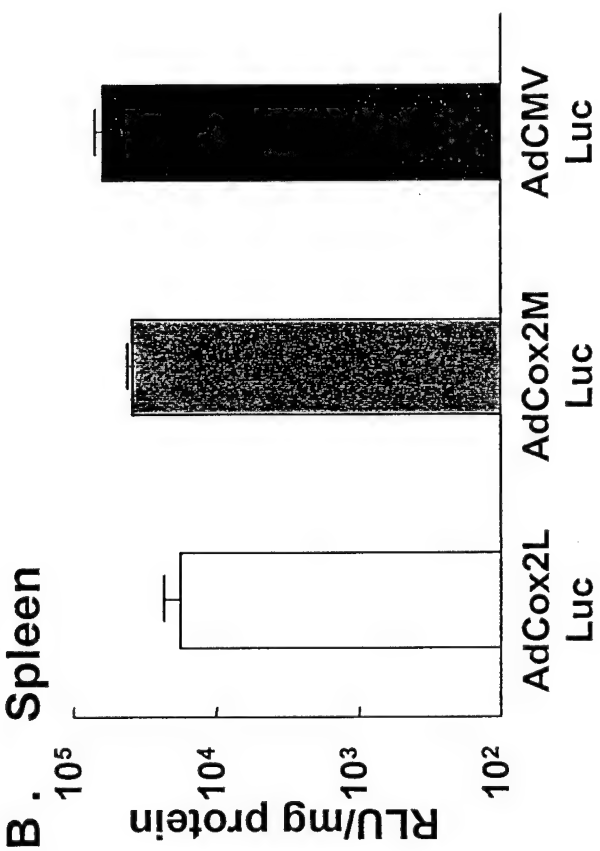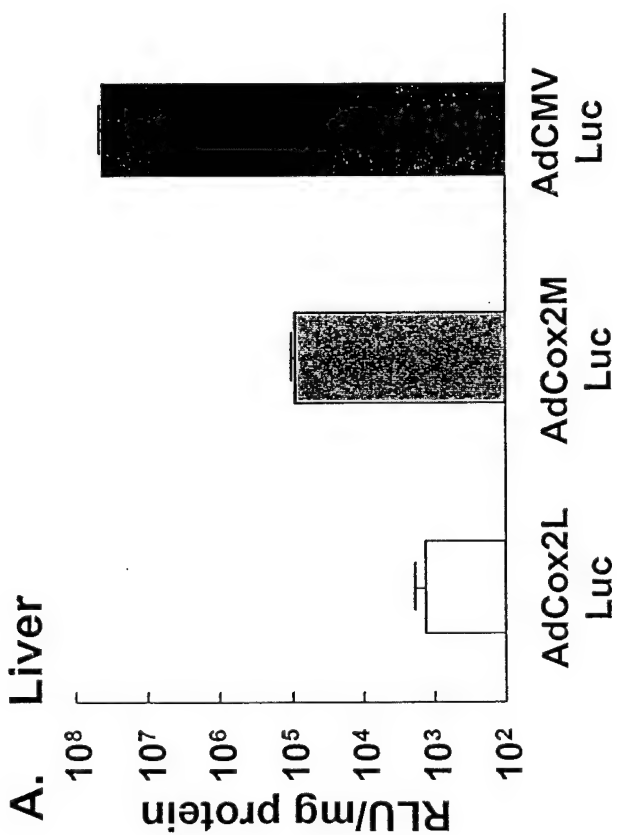
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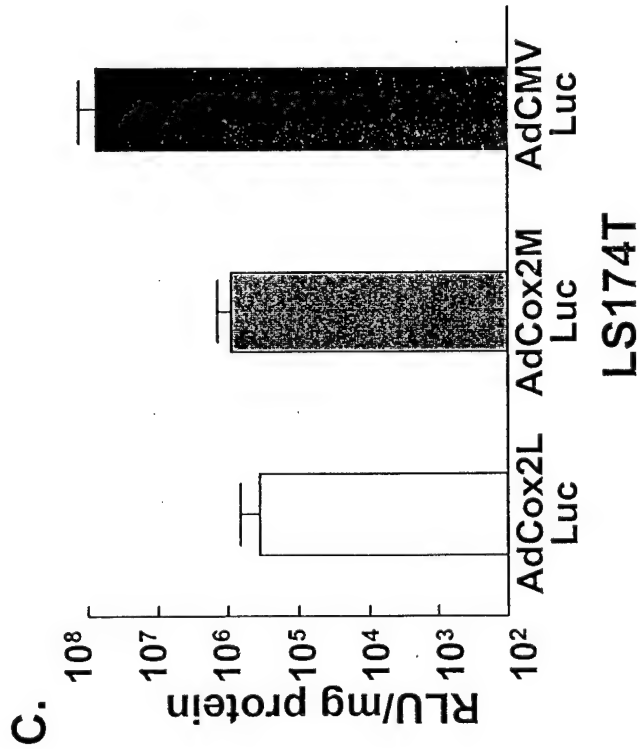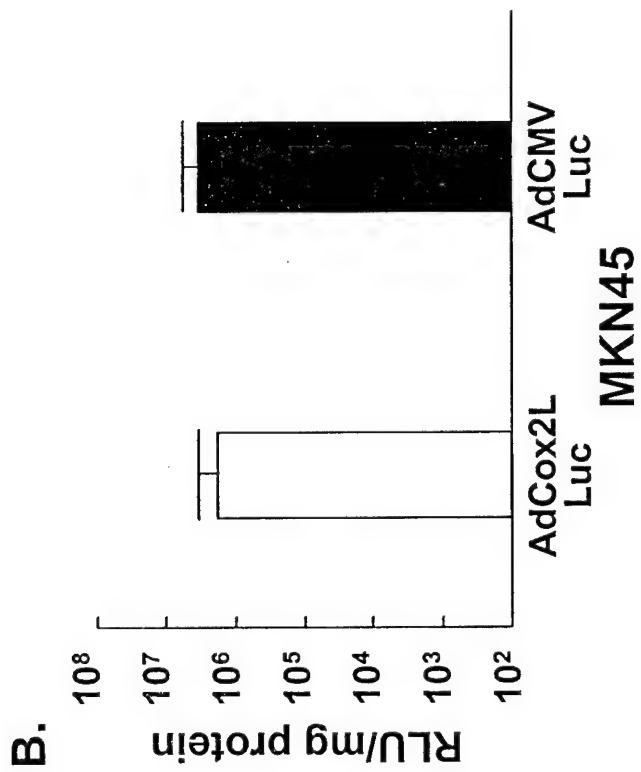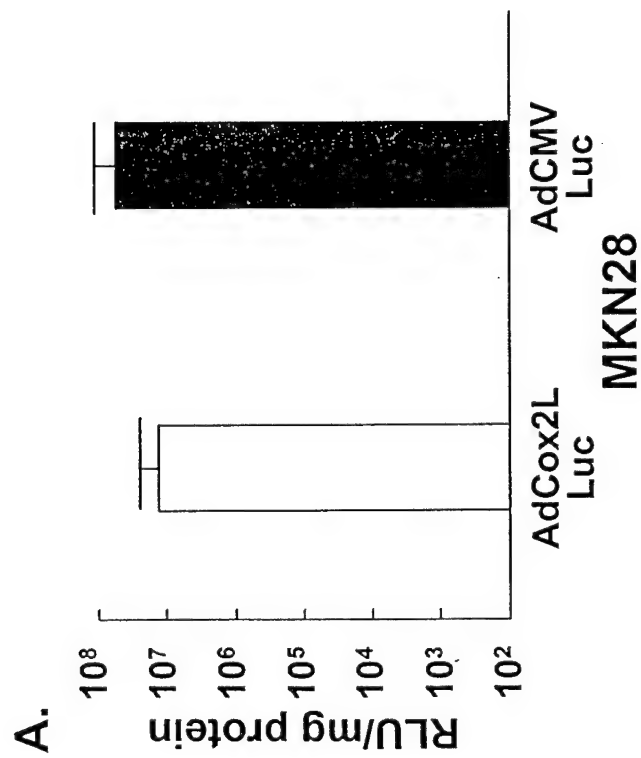
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ΔE1

L-LTR

R-ITR

Adcox2L Luc

-1432　　Long (L)　　+59　　Luciferase

Adcox2L TK

-1432　　Long (L)　　+59　　HSV-tk

Adcox2M Luc

-883　　Medium (M)　　+59　　Luciferase

Adcox2M TK

-883　　Medium (M)　　+59　　HSV-tk

A. COX-2
723 bp →

B. GAPDH
180 bp →

HepG2 Caco-2 Kato3 Huh6 Huh7 MKN28 MKN45 LS174T control A549 H358

A. Adcox2L Luc — MRLU/mg protein

B. Adcox2M Luc — MRLU/mg protein

C. RGDcox2L Luc — MRLU/mg protein

D. RGDcox2M Luc — MRLU/mg protein

A. Liver
RLU/mg protein
10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2
AdCox2L Luc, AdCox2M Luc, AdCMV Luc

B. Spleen
RLU/mg protein
10^5, 10^4, 10^3, 10^2
AdCox2L Luc, AdCox2M Luc, AdCMV Luc

C. Lung
RLU/mg protein
10^6, 10^5, 10^4, 10^3, 10^2
AdCox2L Luc, AdCox2M Luc, AdCMV Luc

D. Kidney
RLU/mg protein
10^5, 10^4, 10^3, 10^2
AdCox2L Luc, AdCox2M Luc, AdCMV Luc

A. MKN28
B. MKN45
C. LS174T

Legend:
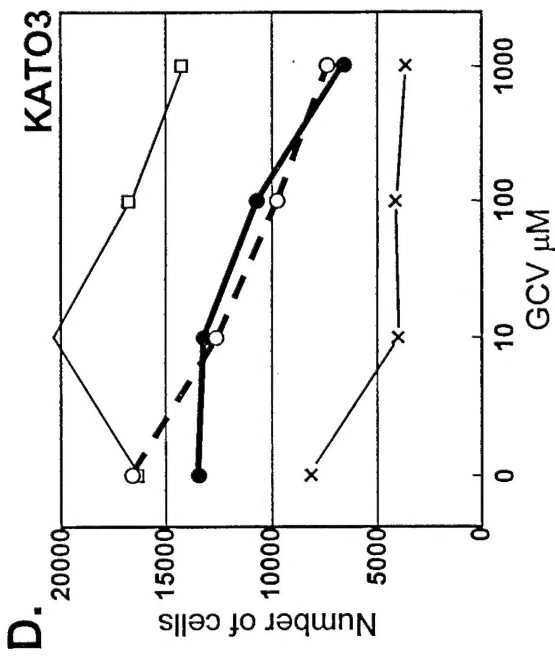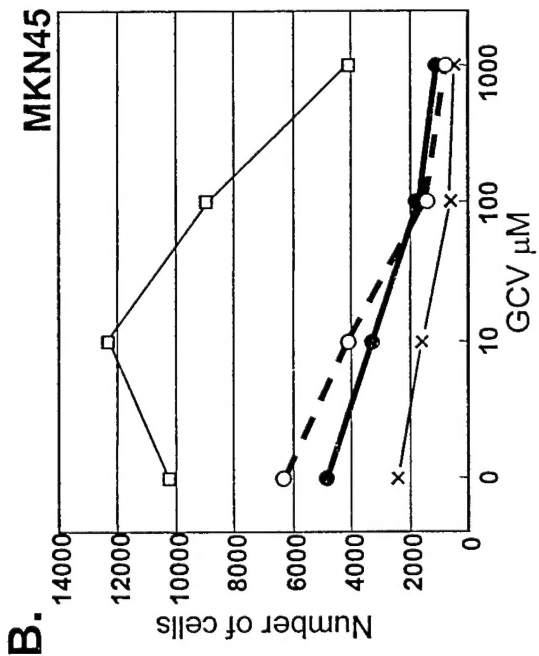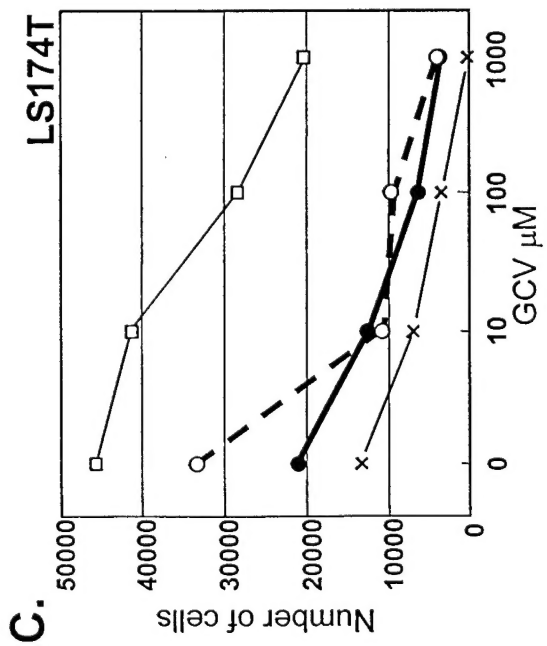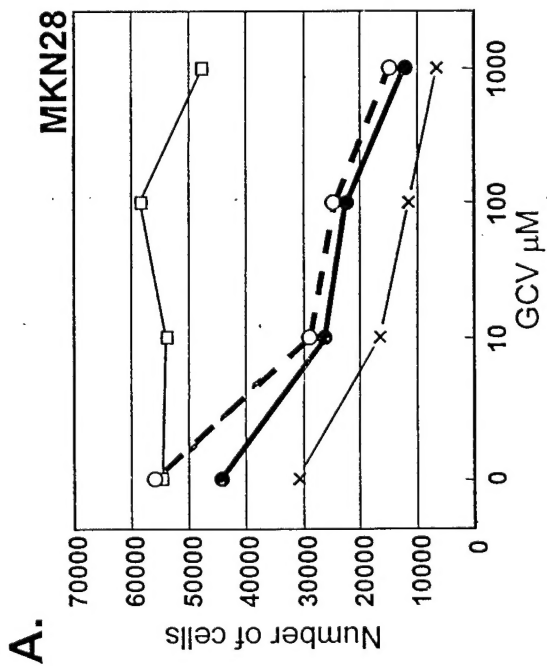- AdGFP
- Adcox2L TK
- Adcox2M TK
- AdCMV TK

A. MKN28
Number of cells (70000, 60000, 50000, 40000, 30000, 20000, 10000, 0)
GCV μM (0, 10, 100, 1000)

B. MKN45
Number of cells (14000, 12000, 10000, 8000, 6000, 4000, 2000, 0)
GCV μM (0, 10, 100, 1000)

C. LS174T
Number of cells (50000, 40000, 30000, 20000, 10000, 0)
GCV μM (0, 10, 100, 1000)

D. KATO3
Number of cells (20000, 15000, 10000, 5000, 0)
GCV μM (0, 10, 100, 1000)